CIRCULAR TEMPLATES AND METHODS

CROSS-REFERENCE TO RELATED APPLICATIONS

Priority is claimed to and this application is a continuation-in-part of U.S. provisional application Serial No. 60/080,198, filed on March 31, 1998.

The present invention is directed to circular templates and methods. More particularly, the present application is directed to the design, synthesis, and methodology of template-directed chemical ligations and oligomerizations of DNA or RNA oligonucleotides and mononucleotides. Further, the present invention is directed to the use of circularized DNA or RNA as circular templates for directing the assembly of reaction substrates. Still further, the present invention is directed to the efficient ligation of short DNA oligonucleotides on circularized DNA templates.

Yet further, the present invention is directed to a method of producing therapeutically active oligonucleotides using small circularized templates. Also, the present invention is directed to an anti-gene method using natural and/or non-natural oligonucleotides for sequence specific binding to double helical DNA using natural and/or non-natural oligonucleotides synthesized in a single chemical step accomplished under aqueous conditions using small circularized DNA as a template.

Moreover, the present invention is directed to chemical ligation and/or oligomerization of DNA on circular DNA templates. Further, the present invention is directed to chemical ligation and/or oligomerization of deoxyoligonucleotides and/or mononucleotides using a circular DNA template as a thermodynamically stable substrate-template complex.

Still further, the present invention is directed to non-enzymatic, template-directed ligation which is particularly advantageous for constructing non-natural, modified oligonucleotides. Further, the present invention is directed to the production of short ODNs with DNA templates. Yet further, the present invention is directed to circular templates and methods adapted from linear template directed reactions but modified by circularizing the template such that the template will display recognition elements on opposing sides of the circular template for complexation with the substrates undergoing reaction.

We report the use of small circular DNA as a triplex-directing template for the highly efficient chemical ligation of oligodeoxyribonucleotides (ODNs) using cyanogen bromide (BrCN). These investigations compared the use of a linear homopyrimidine DNA template (17mer) and a circular pyrimidine-rich DNA template (44mer) for directing the chemical ligation of two homopurine ODNs (6mer + 11mer). The effects of substrate/template ratio, buffer, salt, ionic strength, pH and temperature have been examined in the BrCN activated ligation reactions. The optimal yield of 51% for ligation on the linear template was at pH 6.0, 200 mM $MgCl_2$, 4°C. In contrast, near quantitative ligation on the circular template occurred at higher pH, higher temperature, and showed less dependence on Mg^{2+} concentration (97% yield, pH 7.5, 200 mM $MgCl_2$, 25°C). The relative observed rate of the ligation reaction was a minimum of 35 times faster on the circular DNA template relative to the linear template at pH 7.5, 200 mM $MgCl_2$, 4°C. These investigations reveal that chemical ligation of short ODNs on circularized DNA templates through triplex formation is a highly efficient process over a broad range of conditions.

INTRODUCTION

The shift in emphasis of genome research from mapping to sequencing and functional analysis (1) is placing a high priority on the development of tools for functional genomic studies and impending applications (2). Natural and modified oligodeoxyribonucleotides (ODNs) have long functioned in such roles. They are used as research tools for molecular biology (3), as recognition elements in disease diagnostics (4,5), and are of intense interest as genetic regulators and therapeutic agents (6–9). These expanding applications of ODNs have led us to explore the development of a template-directed approach for the synthesis of ODNs. We report on the use of circular DNA as a template for efficiently directing the ligation of ODNs.

The ability to non-enzymatically direct phosphodiester bond formation of two ODNs in aqueous solution through the action of a phosphate activating reagent and a nucleic acid template was first realized in 1966 (10). Numerous oligonucleotide ligation reactions in duplex- (11–13) and triplex- (14–17) directed systems have been reported since that time. Ligation strategies are advantageous for constructing circular (18–22) and modified oligonucleotides (23–34). Chemically activated template-directed

ligation reactions have also gained interest for their potential role in prebiotic DNA and RNA synthesis (35–38). The higher fidelity of template-directed ligation of short oligonucleotides offers a viable alternative to template-directed mononucleotide oligomerization (39,40). The promising results seen in template-directed ligation reactions in terms of yield and reaction rates (41) has yet to be extended into oligomerization reactions. The obvious challenge for template-directed oligomerizations is to improve the substrate–template association while still allowing product turnover. While we are developing approaches to accomplish both of these objectives, this initial report focuses on a modified DNA template to improve substrate–template association.

An approach was sought for improving substrate binding to a DNA template by maximizing aromatic stacking and hydrogen bonding interactions. A pyrimidine-rich DNA template that binds to reacting substrates through both Watson–Crick and Hoogsteen hydrogen bonding would result in a triplex structure with the reacting homopurine substrates bound as the central strand of the triplex (Fig. 1, top). Further improvement in binding and sequence specificity for purine-rich single-strand DNA has been demonstrated by circularizing the pyrimidine-rich strands of the triplex (42). We report the use of a pyrimidine-rich circular DNA template for directing the chemical ligation of homopurine ODNs (Fig. 1, bottom). The circular DNA template has proved far superior to a single-strand DNA template for directing the chemical activated ligation of two ODNs.

MATERIALS AND METHODS

General experimental

Phosphoramidites including 5-methylcytosine, solid supports and chemicals for DNA synthesis were obtained from Cruachem, Inc. or Peninsula Laboratories. All enzymes were purchased from Boehringer Mannheim, New England Biolabs or Gibco BRL Products. The radiolabeled 5′-[γ-^{32}P]ATP (>6000 Ci/mmol) was obtained from Amersham. All other chemicals were of analytical or HPLC grade. Standard molecular biology techniques were used, if not mentioned otherwise (43). Analytical and preparative HPLC was performed with a Shimadzu LC-600 liquid chromatograph with SPD-6A UV using Varian 150 × 4.6 mm, 5 µm, C18, 90 Å column [acetonitrile–0.1 M triethyl ammonium acetate (TEAA) buffer (pH 7.0) as gradient]. Sep-Pak™ was purchased from Waters. UV measurements were run with a Hitachi U-2000 spectrophotometer. Melting studies were carried out in teflon 1 cm path length quartz cells under nitrogen atmosphere on a Jasco-710 spectropolarimeter equipped with PTC-343 temperature control. Scintillation counting was done on a Wallac 1410 Liquid Scintillation Counter. Gel

Figure 1. Top: triplex resulting from homopurine ligation fragments bound to pyrimidine bases of the circular DNA template (R represents backbone of ligating fragments). Note the use of 5-methylcytidine on the Hoogsteen side of the circular template. Bottom: ribbon graphic of a ligation reaction of two ODNs directed by a circular DNA template.

images were scanned using AGFA-ARCUS II scanner at high resolution, low contrast and imported using Adobe Photoshop 4.0 software. Quantitative analyses of gel images were done with IP Lab Gel using scanned images.

Oligonucleotide synthesis

Oligodeoxyribonucleotides were synthesized on an Applied Biosystems 392 synthesizer using β-cyanoethyl phosphoramidite chemistry (44). Phosphorylation at the 3′-end of the pre-circle oligomer was carried out using modified solid support purchased from Peninsula Laboratories. Cleavage of the solid supports along with the base protecting groups and the phosphate protecting groups were achieved using concentrated ammonium hydroxide. Oligomers were purified by electrophoresis on 20% polyacrylamide gels with 8 M urea and Tris-borate-EDTA (TBE) buffer. The oligonucleotides were isolated from the gels by excision and elution with Tris-EDTA-NaCl (TEN) buffer. The resulting solution was desalted (using Sep-Pak™) and quantified by absorbance at 260 nm using extinction coefficients that were calculated by the nearest neighbor method (45).

Circularization procedure

Linear 3′-phosphorylated oligonucleotide and the complementary homopurine template were combined in a 1:1 ratio (50 mM) with $MgCl_2$ (20 mM) in morpholinoethanesulfonic acid-Et_3N buffer (MES) (250 mM, pH 7.5). After heating to 95 °C, the solution was cooled to 4 °C (19). The reaction was initiated by adding BrCN (500 mM) and allowed to proceed for 24 h at 4 °C. The reaction mixture was quenched with EDTA, lyophilized and redissolved in the loading buffer, and purified by 20% denaturing PAGE. The circular products migrated at 72% the rate of the linear precursor. Circular oligonucleotide **1** was isolated in 60% yield. To verify the product, melting studies were performed on circular oligonucleotide

1 with the complementary single-strand oligonucleotide and compared with the corresponding precircularized, linear oligonucleotide and the complementary single-strand oligonucleotide under identical conditions (see supplementary material). Oligonucleotides were diluted to 3 μM each with 100 mM NaCl, 10 mM $MgCl_2$ in 10 mM MES-Et_3N (pH 7.5). Solutions were heated to 90°C and allowed to cool slowly to room temperature prior to the melting experiments. Absorbance (260 nM) was monitored while temperature was raised at a rate of 0.5°C/min. In all cases the complexes displayed sharp, two-state transitions. Melting temperatures (T_m) were taken to be the temperature of half-dissociation and were obtained from a plot of temperature versus absorbance at 260 nM. Precision in T_m values were estimated from variations of a minimum of two repeated experiments was ±0.5°C. An increase of 11°C in T_m was obtained from the closure of the linear to the circular oligonucleotide **1**. Further verification that **1** was circularized was realized by showing the complete resistance of **1** to cleavage by exonucleases.

Radiolabeling

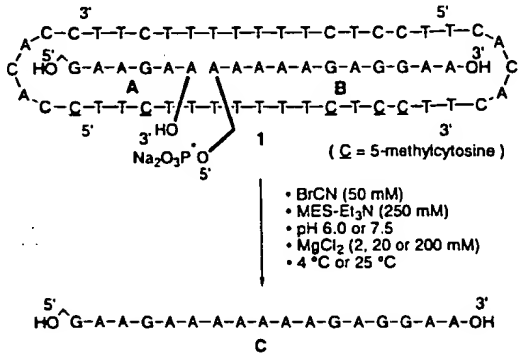
The gel purified oligomer **B** (10 pmol, without 5′-phosphate) was dissolved in 10.4 μl of sterilized, double deionized water. To this was added 2 ml of 10× kinase buffer, 6 μl of [γ-^{32}P]ATP (60 μCi), and 2 μl of T4 polynucleotide kinase (10 000 U/ml) for a total volume of 21.4 μl. Following incubation at 37°C for 3 h, the reaction mixture was heated at 70°C for 10 min and the radiolabeled oligomer purified by chromatography.

Ligation reactions

A 1:1 mixture of radiolabeled oligomer **B** (1 pmol) and ligating fragment **A** (1 pmol) along with template **1** or **2** (2.0 equivalents) was dissolved in 10 μl of MES-Et_3N buffer (500 mM) and 4 μl of the appropriate concentration of $MgCl_2$ (1.0, 0.10 or 0.01 mM) was added. The Eppendorf tube was heated to 95°C and cooled to the reaction temperature. A solution of BrCN (2 μl, 5.0 M in CH_3CN) was added, vortexed, briefly centrifuged and the reaction was allowed to proceed at the desired temperature (4 or 25°C). (20 μl total volume). The final concentration of the ligation reaction components were: buffer (250 mM), $MgCl_2$ (2, 20 or 200 mM) and BrCN (500 mM). At precise intervals, an aliquot (1 μl) of the solution was taken and transferred to a tube containing 48 μl of water and 2 μl of 0.5 M EDTA (pH 7.5). For analysis of shorter reaction times (as fast as 1.0 ± 0.5 s) during the course of the ligation reactions, the entire reaction mixture was immediately frozen by submersion of the Eppendorf tube in a liquid N_2 bath followed by addition of EDTA (20 mM, 1.0 ml) and equilibration to ambient temperature with vortexing. An aliquot (1 μl) of that resulting solution was evaporated to dryness, redissolved in the loading buffer, normalized and subjected to electrophoresis on polyacrylamide gel (8%, 8 M urea). Autoradiography was used to analyze the reaction progression. Product yields were determined from the scanned images of the autoradiograms. The yield was calculated from the densitometry ratio of ligated product **C** relative to the total radioactivity per lane.

RESULTS AND DISCUSSION

Our initial studies of template-directed reactions compared the single ligation of two homopurine ODNs on pyrimidine-rich circular DNA template **1** (Scheme 1) relative to homopyrimidine linear template **2** [d(TTCCTCTTTTTTCTTC)] (43,44). Template **2** was designed for duplex-directed ligation reactions through Watson–Crick hydrogen bonding alone. Circular template **1** was designed with partial incorporation of 5-methylcytidine in order to improve selectivity for the Hoogsteen hydrogen bonding to one side of the circular template (Fig. 1, top) (46–49). Template **1** was synthesized in 60% yield by slight modification of an existing procedure (19).



Scheme 1.

The single ligation reaction was optimized on both circular template **1** and linear template **2** using ligating ODNs **A** (a 6mer) and **B** (an 11mer) (Scheme 1). ODN **B** was 5′-^{32}P-labeled (43) for analysis and quantification of the ligation reactions by autoradiogram densitometry. These investigations focused on the effects of substrate/template ratio, buffer, salt, ionic strength, pH and temperature in the BrCN activated ligation reaction to produce **C** (Scheme 1). Initial ligation experiments of **A** and **B** (1:1, 0.1 μM) on template **1** (2.0 equivalents) at 4°C with BrCN (125 mM) in imidazole–HCl buffer (200 mM, pH 6.0 or 7.0) in the presence of Mg^{2+} (20 mM) or Ni^{2+} (100 mM) afforded ligation product **C** in very poor yield. Greatly improved yields were realized by switching to a 4-morpholinoethanesulfonic acid buffer (MES-Et_3N, 250 mM, pH 6.0 or 7.5) with BrCN (500 mM) in the presence of Mg^{2+}. All ligation reactions were run with the corresponding control reactions containing **A** and **B** with no template to assess for off-template reactions, and **B** with template **2** to assess for any other template-directed reactions. Varying the ratio of ligating fragments **A** and **B** relative to template **1** or **2** from 1:1 to 1:2 (pH 6.0) had negligible effect on the yield of ligation product **C** (see supplementary material).

We anticipated that lower pH, lower temperature and higher ionic strength would result in optimal substrate/template binding (50) to afford the highest yield. This proved true. with linear template **2** as demonstrated by Figure 2, graph I. Optimal conditions with template **2** afforded 51% yield of the ligated product **C** at pH 6.0, 200 mM $MgCl_2$, 4°C (Fig. 2, graph I). At higher pH the yield decreased to 27% (pH 7.5, 200 mM $MgCl_2$, 4°C). Lower ionic strength significantly diminished ligation on linear template **2** (5% yield, pH 7.5, 20 mM $MgCl_2$, 4°C). Higher temperature completely eliminated ligation on template **2** (pH 6.0, 200 mM $MgCl_2$, 25°C) (Fig. 2, graph II).

In contrast to the ligations on linear template **2**, ligation reactions directed on circular template **1** exhibited tolerance to an extended range of reaction conditions. The yield of ligation product **C** was found to be less affected by change in ionic strength on circular template **1** affording 72 and 73% yield at 20 mM and 200 mM $MgCl_2$, respectively (pH 7.5, 4 °C) (Fig. 2, graph I). Surprisingly, circular template **1** afforded better yields at pH 7.5 (73%) than at pH 6.0 (50%) (200 mM $MgCl_2$, 4°C) (Fig. 2, graph I). Increasing the temperature from 4 to 25°C at pH 6.0 or 7.5 resulted in substantially higher yields on cyclic template **1** while eliminating ligation on template **2** (Fig. 2, graphs I and II). Optimum conditions with circular template **1** provided ligated product **C** in near quantitative yield (97%, pH 7.5, 200 mM $MgCl_2$, 25°C) (Fig. 2, graph II).

Attempts were made to assess the difference in the observed rate of formation of ligated product **C** in the BrCN activated reaction on circular template **1** compared to linear template **2**. Conventional autoradiogram densitometric analysis of the amount of ligated product **C** in reaction aliquots from both the single-strand and circular template directed reactions under identical conditions (pH 7.5, 200 mM $MgCl_2$, 4°C) at early time points in the reactions failed to afford a satisfactory reproducible linear correlation. While we are investigating more precise methods for comparing the observed rates of the template directed ligation reactions at early reaction times, a qualitative comparison can be made with the data acquired. A minimum yield of 3% ligation product **C** was required for accuracy by the densitometric analysis methods used. A relative comparison between the earliest reproducible data points (minimum of two independent experiments within ±1% yield) can be compared between the ligation on circular template **1** and linear template **2**. Densitometric analysis of the autoradiogram of aliquots taken at 1.0 ± 0.5 s from the ligation reaction directed by circular template **1** reveal a 39% yield of radiolabeled ligation product **C**. This corresponds to a reaction which is ~53% complete (final yield of 73% **C**). The earliest data affording sufficient product yield in the corresponding linear template **2** directed ligation was obtained at 30 ± 0.5 s, where a 3% yield of **C** was observed. This corresponds to a reaction which is ~11% complete (final yield of 27% **C**). Accounting for experimental error, this results in a minimum difference in the observed rate of ligation product **C** formation of

35 times faster on circular template **1** relative to linear template **2**. The circular template directed ligation not only afforded approximately three times higher product yields relative to the single-strand template under these conditions (73 versus 27%, respectively, graph I), but also affords ligation product at a minimum 35 times faster observed rate.

Figure 2. 3-D bar graphs showing the yield (%) of ligation product C. Graph I shows ligation results at 4°C, pH 7.5 and 6.0 with $MgCl_2$ concentrations of 2, 20 and 200 mM. Graph II shows the same ligation reactions run at 25°C. Data for these graphs was obtained at a reaction time of 3 h. All reactions were reproduced at least twice to afford a % yield error of ±3.

CONCLUSION

A comparative study of the non-enzymatic ligation of two homopurine ODNs on a pyrimidine-rich circular DNA template and a homopyrimidine single-strand DNA template has been accomplished. The optimal conditions for ligation on single-strand template **2** afforded 51% yield. In contrast, near quantitative ligation (97% yield) on circular template **1** occurred at higher pH, higher temperature, and showed less dependence on Mg^{2+} concentration. The observed rate of the ligation reaction was a minimum of 35 times faster on the circular template than the single-strand template. These investigations reveal that chemical ligation of short ODNs on circularized DNA templates through triplex formation is a highly efficient process over a broad range of conditions. This confirms the expected advantage of improving the substrate binding in template-directed ligation reactions by the use of circular DNA templates. The thermodynamic advantage in template binding has allowed for ligation under conditions of higher pH and higher temperatures where the ligation reaction is much more efficient. The possible advantages on the kinetics of the ligation reaction will require further investigations.

The significant yields and reaction rates found in these investigations suggest the high potential for use of circular DNA templates in development of a chemically activated template-directed methodology for the synthesis of homopurine ODNs. Multiple ligations, oligomerizations and reactions of non-natural nucleic acid derivatives are in progress. Extending this methodology beyond purine derivatives for circular template-directed reactions and improving product turnover are under development.

See supplementary material including gel densitometry images and T_m measurements for product characterization below.

ACKNOWLEDGEMENT

The authors gratefully acknowledge partial support of this research by the National Science Foundation (EHR-9108762).

REFERENCES

1 Letovsky,S.I., Cottingham,R.W., Porter,C.J. and Li,P.W.D. (1998) *Nucleic Acids Res.*, **26**, 94–99. See the Human Genome Database (GDB) hosted at Johns Hopkins University (http://www. gdb.org/).
2 Christoffersen,R.E. (1997) *Nature Biotechnol.*, **15**, 483–484.
3 Dean,N.M., McKay,R., Miraglia,L., Geiger,T., Müller,M., Fabbro,D. and Bennett,C.F. (1996) *Biochem. Soc. Trans.*, **24**, 623–629.
4 Cotton,R.G.H. (1993) *Mutat. Res.*, **285**, 125–144.
5 Yershov,G., Barsky,V., Belgovskiy,A., Kirillov,E., Kriendlin,E., Ivanov,I., Parinov,S., Guschin,D., Drobishev,A., Dubiley,S. and Mirzabekov,A. (1996) *Proc. Natl Acad. Sci. USA*, **93**, 4913–4918.
6 Demesmaeker,A.D., Häner,R., Martin,P. and Moser,H.E. (1995) *Acc. Chem. Res.*, **28**, 366–374.
7 Egli,M. (1996) *Angew. Chem. (Engl. Ed.)*, **35**, 1894–1909.
8 Crooke,S.T. and Bennett,C.F. (1996) *Annu. Rev. Pharm. Toxicol.*, **36**, 107–129.
9 Maher,L.J. (1996) *Cancer Investig.*, **14**, 66–82.
10 Naylor,R. and Gilham,P.T. (1966) *Biochemistry*, **5**, 2722–2728.
11 Kanaya,E. and Yanagawa,H. (1986) *Biochemistry*, **25**, 7423–7430.
12 Zuber,G., Sirlin,C. and Behr,Z.P. (1993) *J. Am. Chem. Soc.*, **115**, 4939–4940.
13 Dolinnaya,N.G. and Shabarova,Z.A. (1996) *Russ. Chem. Bull.*, **45**, 1787–1809.
14 Luebke,K.J. and Dervan,P.B. (1989) *J. Am. Chem. Soc.*, **111**, 8733–8735.
15 Luebke,K.J. and Dervan,P.B. (1992) *Nucleic Acids Res.*, **20**, 3005–3009.
16 Dolinnaya,W.G., Pyatrauskene,O.V. and Shabarova,Z.A. (1991) *FEBS Lett.*, **284**, 232–234.
17 Li,T., Weinstein,D. and Nicolaou,K.C. (1997) *Chem. Biol.*, **4**, 209–214.
18 Prakash,G. and Kool,E.T. (1992) *J. Am. Chem. Soc.*, **114**, 3523–3527.
19 Dolinnaya,N.G., Blumenfeld,M., Merenkova,I.N., Oretskaya,T.S., Krynetskaya,N.F., Ivanovskaya,M.G., Vasseur,M. and Shabarova,Z.A. (1993) *Nucleic Acids Res.*, **21**, 5403–5407.
20 Rubin,E., Rumney,S.,IV, Wang,S. and Kool,E.T. (1995) *Nucleic Acids Res.*, **23**, 3547–3553.
21 Herrlein,M.K., Nelson,J.S. and Letsinger,R.L. (1995) *J. Am. Chem. Soc.*, **117**, 10151–10152.
22 Xu,Y. and Kool,E.T. (1998) *Nucleic Acids Res.*, **26**, 3159–3164.
23 Zielinski,W.S. and Orgel,L.E. (1987) *Nature*, **327**, 346–347.
24 von Kiedrowski,G., Wlotzka,B., Helbing,J., Matzen,M. and Jordan,S. (1991) *Angew. Chem. (Engl. Ed.)*, **30**, 423–426.
25 Dolinnaya,N.G., Sokolova,N.I., Ashirbekova,D.T. and Shabarova,Z.A. (1991) *Nucleic Acids Res.*, **19**, 3067–3072.
26 Rumney IV,S. and Kool,E.T. (1992) *Angew. Chem. (Engl. Ed.)*, **31**, 1617–1619.
27 Sawai,H., Totsuka,S., Yamamoto,K. and Ozaki,H. (1988) *Nucleic Acids Res.*, **26**, 2995–3000.
28 Gryaznov,S.M. and Letsinger,R.L. (1993) *J. Am. Chem. Soc.*, **115**, 3808–3809.

29 Herrlein.M.K. and Letsinger.R.L. (1994) *Nucleic Acids Res.*, **22**, 5076–5078.
30 Pitsch.S., Krishnamurthy.R., Bolli.M., Wendeborn.S., Holzner.A., Minton.M., Lesueur.C., Schlönvogt.I., Jaun.B. and Eschenmoser.A. (1995) *Helv. Chim. Acta*, **78**, 1621–1634.
31 Böhler.C., Nielsen.P.E. and Orgel.L.E. (1995) *Nature*, **376**, 578–581.
32 Bruick.R.K., Dawson.P.E., Kent.S.B.H., Usman.N. and Joyce.G.F. (1996) *Chem. Biol.*, **3**, 49–56.
33 Schmidt.J.G., Christensen.L., Nielsen.P.E. and Orgel.L.E. (1997) *Nucleic Acids Res.*, **25**, 4792–4796.
34 Luo.P., Leitzel.J.C., Zhan.Z.-Y.J. and Lynn.D.G. (1998) *J. Am. Chem. Soc.*, **120**, 3019–3031.
35 Li.T. and Nicolaou.K.C. (1994) *Nature*, **369**, 218–221.
36 Bag.B.G. and von Kiedrowski.G. (1996) *Pure Appl. Chem.*, **68**, 2145–2152.
37 Rohatgi.R., Bartel.D.P. and Szostak.J.W. (1996) *J. Am. Chem. Soc.*, **118**, 3332–3339.
38 Bolli.M., Micura.R. and Eschenmoser.A. (1997) *Chem. Biol.*, **4**, 309–320.
39 James.K.D. and Ellington.A.D. (1997) *Chem. Biol.*, **4**, 595–605.
40 Harada.K. and Orgel.L.E. (1994) *J. Mol. Evol.*, **38**, 558–560.
41 Federova.O.A., Gottikh.M.B., Oretskaya.T.S. and Shabarova.Z.A. (1996) *Nucleosides Nucleotides*, **15**, 1137–1147.
42 Kool.E.T. (1997) *Chem. Rev.*, **97**, 1473–1487.
43 Sambrook.T., Fritsch.E.F. and Maniatis.T. (1989) *Molecular Cloning: A Laboratory Manual*, (2nd ed.), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
44 Beaucage.S.L. and Caruthers.M.H. (1981) *Tetrahedron Lett.*, **22**, 1859–1862.
45 Borer.P.N. (1975) In Fasman.G.D. (ed.), *Handbook of Biochemistry and Molecular Biology*, CRC Press, Cleveland, OH, 3rd ed. vol. 1, p. 589.
46 Plum.G.E., Park.Y.-W., Singleton.S.F., Dervan.P.B. and Breslauer.K.J. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 9436–9440.
47 Xodo.L.E., Manzini.G., Quadrifoglio.F., van der Marel.G.A. and van Boom.J.H. (1991) *Nucleic Acids Res.*, **19**, 5625–5631.
48 Hunziker.J., Priestley.E.S., Brunar.H. and Dervan.P.B. (1995) *J. Am. Chem. Soc.*, **117**, 2661–2662.
49 Soyfer.V.N. and Potaman.V.N. (1996) *Triple-Helical Nucleic Acids*, Springer-Verlag, New York.
50 Saenger.W. (1984) *Principles of Nucleic Acid Structure*, Springer-Verlag, New York, pp. 116–158.

SUMMARY

The present invention is directed to a new, cost efficient, "green process" for the large scale synthesis of homopurine oligodeoxyribonucleotides (ODNs). The therapeutic and diagnostic applications of homopurine ODNs for antisense and antigene approaches makes large scale ODN synthesis (gram to kilogram quantities) of primary importance. The method is designed to far exceed all present means for synthesizing ODNs in regard to production scale, cost, efficiency and environmental impact. The estimated cost of ODN synthesis by the proposed methodology is approximately 0.05% the cost using conventional solid support chemistry. Conventional ODN synthesis techniques also produce considerable waste and environmentally harmful byproducts. In contrast, the proposed methodology produces little waste and the byproducts are relatively harmless (NaBr, HBr, CO_2 and NH_3 in water). This cost efficient green process can be accomplished due to the simplicity of the required starting materials and reagents and the aqueous based nature of the reactions.

The general method targets the use of a DNA template directed reaction to oligomerize unprotected mononucleotides with cyanogen bromide (BrCN) and a divalent metal salt ($MgCl_2$ or $CaCl_2$) in water. The goal of the proposed research is to systematically modify the DNA template for optimal efficiency in directing oligomerization to produce sequence defined homopurine ODNs. This will be accomplished with circular DNA templates having attached, "capped" primers that act to preorganize and cooperatively facilitate oligomerization while preventing primer-substrate reaction. This will provide a stable template for extended use. Methods will be developed for large scale ODN synthesis that allow high turnover for multiple cycles of oligomerization reactions to be performed on the same template.

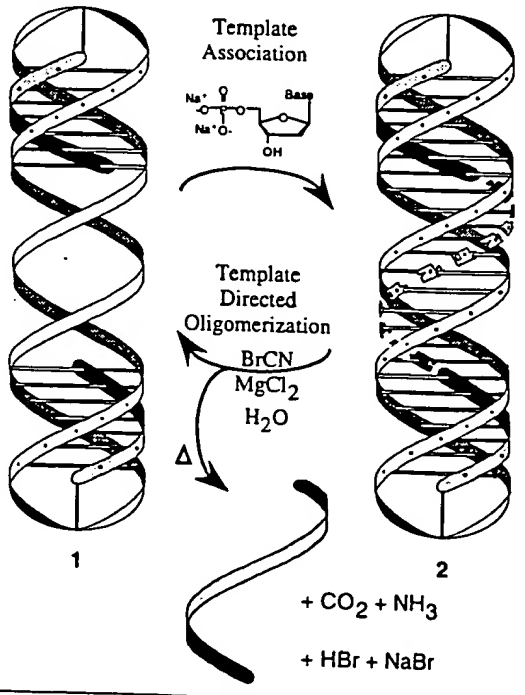
The foundational research established in this invention allow the future extension of this methodology to large scale synthesis of *modified* DNA or RNA oligonucleotides which are required for biodelivery and biostability in diagnostic or therapeutic applications. Development of the basic methodology provides for extension to a variety of non-nucleic acid oligomerization processes for controlled synthesis of functional oligomers.

Introduction

This proposal outlines the development of a new, cost efficient "green process"[1] for the large scale synthesis of homopurine oligodeoxyribonucleotides (ODNs).[2] The therapeutic[3] and diagnostic[4] applications of homopurine ODNs for antisense and antigene approaches makes large scale ODN synthesis (gram to kilogram quantities) of primary importance.[5] The method is designed to far exceed all present means[6] for synthesizing ODNs in regard to production scale, cost, efficiency and environmental impact. Compared to conventional methods for ODN synthesis it is anticipated to be **0.05% the cost**[7] and produce **only** environmentally harmless byproducts (NaBr, HBr, CO_2 and NH_3 in water). This can be accomplished due to the simplicity of the required starting materials and reagents and the aqueous based nature of the reactions. The foundational research established in this proposal will allow the future extension of this methodology to large scale synthesis of modified DNA or RNA oligonucleotides which are required for biodelivery and biostability in diagnostic or therapeutic applications.[8] Development of the basic methodology will also allow future extension to a variety of non-nucleic acid oligomerization processes for controlled synthesis of functional oligomers.

The general method targets the use of a DNA template directed reaction to oligomerize unprotected mononucleotides with cyanogen bromide (BrCN) and a divalent metal salt ($MgCl_2$ or $CaCl_2$) in water (Figure 3). The effectiveness of the template directed oligomerization will be improved by circularizing the DNA template to allow a triplex directed oligomerization having the mononucleotides as the central strand (Fig. 1). Further modifications of this stable DNA template will be introduced to enhance template "preorganization" and reaction efficiency as the research progresses. Such modifications include the use of attached "primers" that will not be covalently incorporated into the oligomer being synthesized (**1**, Figure 3). Large scale ODN synthesis (estimated at several kilogram/day potential) can be realized since the circular DNA template appears stable to the reaction conditions. Methods will be developed to allow for multiple cycles of oligomerization reactions to be performed on the same template for "catalytic" template use.

Figure 3



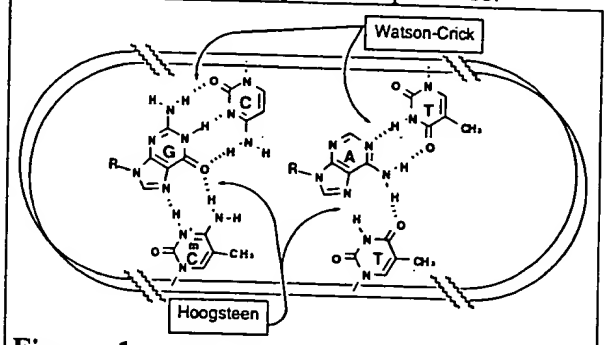


Figure 1. Magnified view of the purine substrates bound via triplex formation to the circular DNA template (^mC = 5-methylcytosine).

Relative to a single strand template directed ligation reaction, triplex directing template **1** will greatly improve the association of the mononucleotide substrates to the template through both Watson-Crick and Hoogsteen hydrogen bonding (Fig. 1). The improved template association allows for higher reaction temperatures which improves the kinetics of the oligomerization reaction and results in a highly efficient synthetic method. Recycling of any nucleotide starting materials will be facilitated by the hydrolytic reversibility of phosphate activation with BrCN. The additional "green" aspects of this process include the minimal reagent requirement (BrCN only), the hydrolytic breakdown of BrCN to NaBr (Na from the nucleotide salt components), HBr, CO_2 and NH_3 as the only waste byproducts, and the aqueous based nature of

the chemistry. In total, this green process could be revolutionary as a commercial method for large scale ODN synthesis in terms of cost,[7] efficiency and environmental impact.

Background

The anticipated completion of the human genome sequencing project in five years has placed an emphasis on methods which will allow functional analysis of the human genome[6,9] and prepare the way for diagnostic[3] and therapeutic[4] approaches for disease analysis and treatment. One of the most direct means to mediate protein production and genetic transformation is through antisense and antigene approaches.[10] One of the key limitations which will need to be addressed in bringing this technology to diagnostic and therapeutic application is the ability to produce large scale (gram to kilogram) quantities of desired natural and non-natural oligonucleotides. To our knowledge there have been no "green processes" reported which address this need in a cost effective manner.

The state-of-the-art techniques for oligonucleotide synthesis using well established, automated, solid-phase chemistry are based on elegant phosphoramidite,[11] phosphite-triester,[12] or H-phosphonate approaches.[13] Recent advances in solid-phase synthesizers allow multi-gram scale (up to 5 mmol) production of pure ODNs.[5a] More recently, Ravikumar and coworkers at Isis Pharmaceuticals reported the use of Pharmacia's OligoProcess synthesizer to produce kilogram quantities of pure phosphorothioate ODNs for clinical trials.[14] While very little detail was given in this report, Pharmacia's instruments have made extensive progress in overcoming traditional drawbacks of solid support synthesis such as limited reaction rate and yield due to limited permeability and steric hindrance of the heterogeneous reaction mixture. But of far greater concern is the high cost and the environmental impact of high volume waste that is generated by large scale, multistep synthesis of oligomers by solid support approaches. The use of specially synthesized supports, multiple protecting groups, specialized activated derivatives and reagents for couplings and oxidations, the requirement for anhydrous conditions, repeated capping of unreacted groups, and multiple washing cycles results in a high cost for reagents, operation, maintenance and waste disposal. The overall economic and environmental impact is therefore less attractive than a "green process" that would allow the use of cheap starting materials, few reagents, aqueous based chemistry, produce little waste, and allow recycling of unaltered starting materials.

An alternative to solid support chemistry has been the development of solution based methods for large scale ODN synthesis under homogeneous conditions.[15] The most attractive approaches incorporate the advantages of solid supports by performing the synthesis on a high molecular weight polymer for ease of purification steps through size exclusion methods.[16] However, the polymer is soluble to maintain reaction homogeneity so that reaction efficiency is high and large scale reactions can theoretically be achieved. To our knowledge, the largest scale ODN synthesis using this approach has been up to hundreds of milligrams.[17] Although larger scale reactions can theoretically be performed by these solution based approaches, the economic disadvantages of starting material and reagent costs, complex protecting group requirements and high volume solvent use and waste disposal make these approaches as environmentally and economically unattractive as the solid support methods.

An additional approach for ODN synthesis is through enzymatic oligomerizations.[18] This approach is appealing in terms of avoiding the costly starting materials and the waste disposal problems, but the potential for large scale ODN synthesis is severely limited by several factors. The overexpression of enzymes is a tedious and expensive multistep process which requires time and complicated purification strategies. If enough enzyme could be produced to accomplish kilogram scale ODN synthesis, the expense would likely prove too prohibitive. In addition, while the enzymatic oligomerization reaction itself is efficient, the purification of the desired product is again a multistep, laborious and expensive process. An additional limitation to enzymatic approaches is the inability to produce modified ODNs. Only naturally occurring ODNs can be synthesized enzymatically. The use of ODNs for diagnostic and therapeutic applications requires modified, non-natural derivatives in order to afford biodelivery and biostability characteristics to the ODNs.[8] The economic prohibitions and limitation to natural ODNs by an enzymatic based approach make it unattractive for large scale ODN synthesis of biomedicinal utility.

A highly attractive approach to ODN synthesis is through non-enzymatic, template directed ligations and oligomerizations. The ability to non-enzymatically direct phosphodiester bond formation of two oligonucleotides in aqueous solution through the action of a phosphate activating

reagent and a nucleic acid template was first realized in 1966.[19] Since that time numerous oligonucleotide ligation reactions have been reported in duplex directed systems with single strand DNA templates, where Watson-Crick hydrogen bonding affords the substrate-template association.[20,23c] ODN ligations have also been reported in triplex directed systems with double strand templates, where Hoogsteen hydrogen bonding of homopyrimidine ligation substrates to the homopurine strand of a homopryrimidine-homopurine Watson-Crick duplex affords the substrate-template complex.[21] Non-enzymatic, template directed ligation strategies are particularly advantageous for constructing non-natural, modified oligonucleotides.[22] This includes the synthesis of small, circular DNA through the template directed circularization of linear ODNs.[23]

Chemically activated, template directed ligation and oligomerization reactions have gained interest for their potential role in prebiotic DNA and RNA synthesis.[24,25,26] This area of research has contributed the most significant progress in regard to product turnover for a more "catalytic" use of the templates. However, to our knowledge, application to large scale ODN synthesis has not been an addressed objective. While elegant systems have been developed to study template directed oligomerization, the low yield of oligomerization reactions and requirement for activated nucleotide monomers which suffer from hydrolytic degradation[27] and side reactions[28] limit the synthetic utility of existing approaches for large scale ODN synthesis.

The higher association of short ODNs with DNA templates has resulted in numerous reports of template directed ligation reactions of short ODNs as a less challenging alternative to template directed mononucleotide oligomerizations.[29] Yields as high as 85% have been reported for triplex template directed ligation reactions, although limited to the ligation of longer ODN substrates (two 12-mers to afford a 24-mer, GC content = 50%).[24a] By comparison, the method presented in this proposal affords quantitative yields in a 6-mer plus 11-mer ligation (GC content = 29%).

A largely unexploited potential for template directed oligonucleotide synthesis has been in the development of large scale (gram to kilogram) production of ODNs. This potential has likely gone untapped due to the poor turnover rate resulting in inefficient template utilization.[30] One unique approach to this problem was the "rolling circle DNA synthesis" by Kool[31] and others,[32] where a single strand, circular DNA template has been used for the enzymatic synthesis of extremely long, single strand DNA products composed of multiple copies of the circular template sequence. While this approach has the potential for large scale ODN synthesis, it will be limited by the need for polymerases and restriction enzymes and the limitation to natural ODN synthesis.

This proposal details an approach for improving the thermodynamics of substrate binding to a DNA template by maximizing aromatic stacking and hydrogen bonding interactions. A pyrimidine-rich DNA template which binds to reacting purine substrates through both Watson-Crick and Hoogsteen hydrogen bonding results in a triplex structure with the reacting homopurine substrates bound as the central strand of the triplex (Fig. 1). Further improvement in binding and sequence specificity for purine-rich single strand DNA has been demonstrated by circularizing the pyrimidine-rich strands of the triplex.[33] Additional components will be introduced to further enhance substrate association and regiocontrol in template binding. Regiocontrol will be enhanced through incorporation of modified cytidine (C) derivatives to one side of the circular DNA template (section I.B.3). Cytidine protonation is required for Hoogsteen binding in the C^+•GC triplet.[34] These C-derivatives [*i.e.*, 5-methylcytidine (^{Me}C)[35] or pseudoisocytidine (^{pi}C)[36] and other derivatives [37]]will control which side of the circular DNA template will bind in the Hoogsteen mode by controlling cytidine protonation [by lowering the pK_a (^{Me}C) or having a "permanently protonated" C-derivative (^{pi}C)] (Fig. 1). The incorporation of primers (**1**, Figure 3) at each end of the circular template will "preorganize" the template for substrate binding through triplex formation (section I.D.2-4). These primers will initiate regiocontrol by establishing which side of the template will bind in the Hoogsteen mode. This is anticipated to be propagated through cooperative stacking and steric factors which will favor homogeneity in directional alignment of substrates on the preorganized template. Covalent attachment of these primers to the template will afford a highly stable, preorganized template for optimal substrate binding (section I.D.4). The primers will be "capped" in order to prevent their covalent incorporation into the ODN being produced on the template (section I.D.3). These aspects, combined with the template stability to the BrCN activated reaction conditions (Preliminary Results and section I.A), will allow multiple cycles of template use. Development of high turnover reaction methods (sections I.C and II.A-B) will allow the "catalytic" use of this template for large scale production of homopurine ODNs.

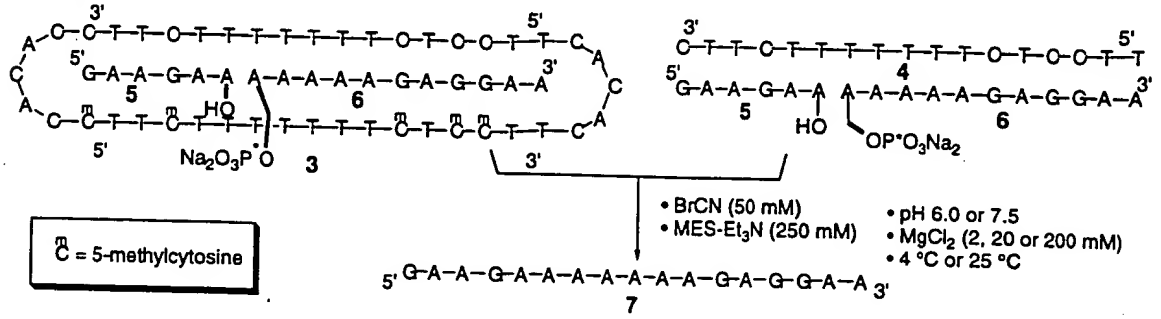
The BrCN activated chemistry for ligation and oligomerization of the template bound substrates has already been proven by the quantitative ligation yield of a short hexadeoxyribonucleotide (6-mer) with an 11-mer (Preliminary Results). The reaction is fast (nearly complete in <1 min) and can be accomplished under aqueous conditions from cheap, commercially available starting monomers which are stable and require no protecting groups for the oligomerization and/or ligation process. The approximate 1,850-fold savings over conventional solid support approaches in material costs[7] and the alleviation of waste concerns could revolutionize industrial large-scale synthesis of diagnostic and therapeutic homopurine oligonucleotides once these initial investigations document the potential of this methodology.

Preliminary Results

Initial investigations have attempted to assess the potential for the use of circular DNA templates to nonenzymatically direct the synthesis of short oligodeoxyribonucleotides (ODNs). The results obtained to date are unprecedented in the field in terms of yield and reaction conditions. Quantitative yields have been realized in ligation reactions with very short ODNs (5-mers and 6-mers) on the circular DNA templates at 25 °C, pH 7.5.

The first investigations focused on determining the effectiveness of circular DNA as a template to direct phosphodiester bond formation between two ODNs (*i.e.*, ligation).[38] These studies compared the use of circular DNA template **3** to single strand DNA template **4**, which directs ligation through Watson-Crick hydrogen bonding to ligating oligonucleotides **5** and **6** (Scheme 2). Ligation directed by the circular DNA template was anticipated to be more efficient due to the improved binding affinities expected through both Watson-Crick and Hoogsteen hydrogen bonding to the ligating fragments. The effects of various parameters were studied in the cyanogen bromide (BrCN) activated ligation reaction including the substrate/template ratio, buffer, salt, ionic strength, pH and temperature. The optimal conditions for ligation on the linear template afforded 51% yield of ligated product **7** (pH 6.0, 200 mM $MgCl_2$, 4 °C).[39] In contrast, near quantitative ligation on the circular template occurred at higher pH, higher temperature, and showed less dependence on Mg^{2+} concentration (≥97% yield, pH 7.5, 200 mM $MgCl_2$, 25 °C). The relative rate of the ligation reaction was found to be approximately 23 times faster on the circular DNA template relative to the linear template (pH 7.5, 200 mM $MgCl_2$, 4 °C). These investigations revealed that chemical ligation of short ODNs on circularized DNA templates through triplex formation is a highly efficient process over a broad range of conditions. The quantitative nonenzymatic ligation of two short ODNs (6-mer + 11-mer) is unprecedented and represents the tremendous potential for this method.

Scheme 2.



A more thorough analysis of the effect of the circular template on the ligation reaction is underway. The template-substrate complex is being analyzed with a combination of melting temperature (T_m) analysis, CD spectroscopy, and differential scanning calorimetry (DSC). These studies will allow the nature of the template-substrate interaction to be more clearly understood. It is anticipated that the circular template binds the reacting ODNs more tightly than the corresponding single strand template effectively lowering the entropy of the ligation reaction through tighter "preorganization" of the reacting ends (*i.e.*, less "fraying" at the ends of the ODNs on the template). The T_m analysis of the circular template with the two ligating substrates **5** and **6** compared to the single strand template with **5**and **6** under the conditions for the ligation reactions has confirmed the expected tighter binding with the circular template. At 200 mM $MgCl_2$, both the

circular template and the single strand template show melting above 25 °C (circular template-substrate complex: T_m = 58 °C; single strand template-substrate complex: T_m = 38 °C). However, only the circular template affords ligation product at 25 °C. At 4 °C, both templates should have the substrate ODNs bound, yet the circular template still reveals superior templating properties based on ligation efficiency (both yield and reaction rate). This may be a result of more "fraying" of the ODN substrates on the single strand template, or perhaps better conformational positioning of the reacting ends on the circular template. The CD analysis of **5** and **6** with no template, **5** and **6** with single strand template **4**, and **5** and **6** with circular template **3** are being analyzed to determine the degree of helicity as a measure of conformational preorganization with the two templates. This will provide information regarding the overall structural rigidity between the two template-substrate complexes. Lastly, DSC experiments are being planned in order to parse out the enthalpic and entropic contributions to the template-substrate binding complexes with the two templates **3** and **4**. These studies will be conducted in conjunction with Professor Wes Stites (University of Arkansas) whose expertise is in the area of analyzing protein dynamics with DSC. Information from these experiments is anticipated to reveal exciting details regarding the difference in substrate preorganization for ligation on the two templates.

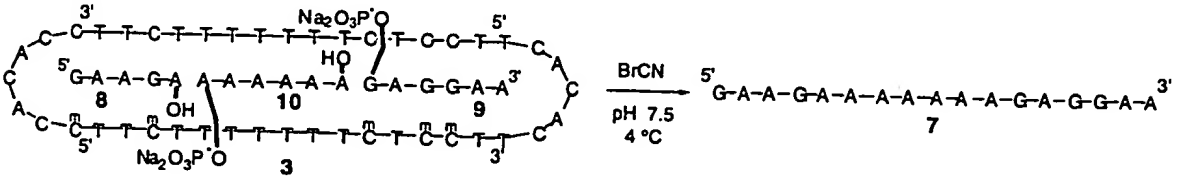
DNA template stability analysis is in progress (section I.A). Initial experiments have involved ambient temperature ^{1}H NMR analysis of D_2O solutions of the dinucleotides (CpC and TpT) with BrCN at various concentrations. To date, these experiments have revealed no change to the dinucleotides which represent the key DNA components of the circular DNA template. Other nucleotide components have yet to be assessed; however, the non-substrate binding, "looped region" of the circular template will be replaced by non-nucleic acid components in future experiments (section I.D.4). As future template designs evolve, necessary control experiments will assess the stability of the various components using this ^{1}H NMR analysis approach and that described below (section I.A). Should any reaction be detected in ongoing experiments, the optimal BrCN concentrations for minimization of side reactions will be determined. The hydrolytic reversibility of any modifications to the template components will be thoroughly examined. Any optimization in the reaction conditions or workup processes will be derived from these studies.

The initial studies of ligation reactions revealed a dependence of the ligation efficiency on the particular divalent metal used in the reaction. A more thorough analysis of the effect of various divalent metals on the BrCN activated ligation reaction is in progress.[40] Calcium ($Ca(NO_3)_2$) and magnesium ($MgCl_2$) appear far superior in promoting the ligation reaction than any other divalent metal examined [including $BaCl_2$, $MnCl_2$, $NiCl_2$, $CoCl_2$, $CuCl_2$, $ZnCl_2$, and $Fe(NH_4)_2(SO_4)_2$.] The effectiveness of the metal appears pH dependent and somewhat temperature dependent. Completion of the study of divalent metals is underway with an examination of the effect of the anion component of the metal salts. At this point, there appears to be a minor difference between ($Ca(NO_3)_2$) and $CaCl_2$. These investigations are leading to optimized conditions for extension to multiple ligation and oligomerization reactions.

The use of a carbodiimide (EDCI) as the activating reagent for ligation has also been examined.[41] It was found that yields are approximately equivalent on the circular DNA template directed reactions, although the reactions are significantly slower. These investigations are providing interesting information regarding template-substrate association as a result of the thermodynamic controlled nature of the EDCI reactions versus the kinetic controlled BrCN reactions. Analysis of these reactions is still in progress and will be reported soon.[41]

Although multiple ligations have not been investigated yet, the conditions used in the initial single ligation reactions on a circular DNA template have been applied to a double ligation reaction (Scheme 3). These conditions are completely unoptimized and therefore will likely represent the lowest ligation efficiency. The double ligation of 5-mer **8** + 6-mer **10** + 6-mer **9** afforded full length 17-mer oligonucleotide **7** in 24% yield (Scheme 3). Again, the nonenzymatic ligation of

Scheme 3.



three oligonucleotides of such short length is unprecedented. This initial result represents tremendous potential for this methodology.

I. Multiple Ligations/Oligomerizations

A. Circular DNA Template Fidelity. The stability of the circular DNA template is presently under examination to certify its fidelity under the BrCN activated ligation conditions. The ^{1}H NMR analysis experiments of the template nucleotide components previously described (Preliminary Results) are ongoing. As discussed, any optimization of reaction conditions will be derived from these studies. Other experiments include submitting ^{32}P labeled circular template **3** bound to triplex forming oligonucleotide (TFO) **7** to successive 5 minute rounds of the BrCN activated ligation conditions reported (see Preliminary Results). Aliquots of each successive round are being examined by PAGE autoradiography. Any degradation of the template will be quantified by densitometry analysis of the resulting autoradiograms. No degradation of the template has been detected to date. Completion of these studies will allow complete documentation of the fidelity of the presently used circular template. These initial investigations are helping to develop stability testing procedures which will be applied to each new modified template introduced in the course of these studies.

Should any significant degradation of the template occur that is not overcome through optimized reaction conditions, modification of the template will also be examined. A particularly appealing modification (one which will be examined in the future regardless of template stability) will be a peptide nucleic acid (PNA) circular template.[43] PNA[44] would be anticipated to show greater stability to electrophilic activating (or acylating-type) reagents such as BrCN having neither a phosphodiester moiety or a glycosidic bond. The high or complete pyrimidine content of the circular DNA template alleviates the depurination pathway for degradation. Any pyrimidine addition products with BrCN are anticipated, and to date experimentally appear to be hydrolytically reversible processes, if occurring. No experiments have revealed any sign of template modification under the BrCN ligating reaction conditions.

B. Optimization of a Double Ligation. The preliminary attempt at a double ligation of a 5-mer + 6-mer + 6-mer afforded 24% yield of the resulting 17-mer (Preliminary Results). This was a completely unoptimized reaction showing a great deal of promise. Studies are continuing to optimize this reaction.[39] A variety of reaction variables are being examined including pre-equilibrium conditions (heating/cooling cycles and times), the ratio of activating

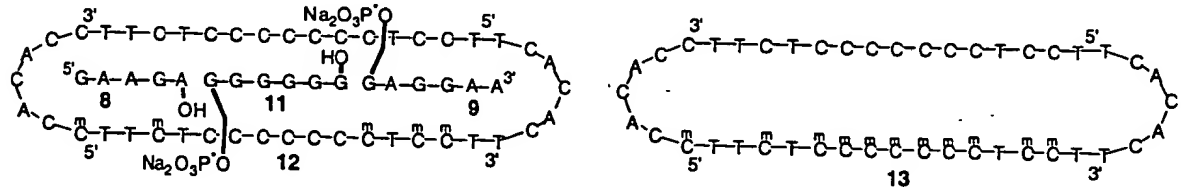
reagent (BrCN) to ligating fragments, sequential additions of BrCN over the reaction course, the use of alternative activating reagents (*i.e.*, carbodiimides such as EDCI), and the concentration of ligating fragments relative to template. Along with these, four additional, primary variables will be examined: (1) the effect of ionic strength and divalent metal, (2) the effect of 5'-phosphate vs. 3'-phosphate in the ligating fragments, (3) the effect of base content of the ligating fragments, and (4) the effect of the length of the ligating fragments.

1. Ionic Strength and Divalent Metals. Initial results from single ligation reactions revealed that ionic strengths of 20-200 mM $MgCl_2$ were optimal. Further studies have shown $Ca(NO_3)_2$ to be equally as effective as $MgCl_2$ in the single ligation reactions. These factors will be examined in the double ligation reaction. Preliminary results reveal that 0.5 M NaCl has minimal benefits on the double ligation reaction.

2. 5'-Phosphate vs. 3'-Phosphate. Some reports reveal a difference in ligation efficiency based on whether the phosphate is on the 5'-end or the 3'-end of the ligating oligonucleotides.[29b] One might expect a less steric approach of the 5'-OH on a 3'-phosphate. Preliminary data from our laboratory suggests that little difference exists in single ligation reactions with the phosphate at either the 3'- or the 5'-end of short ODNs. This will be examined in the context of the double ligation reaction as well. The required 5'-phosphate is obtained through enzymatic phosphorylation or chemical techniques.[45] The 3'-phosphate is obtained by using commercially available modified solid support linkers for automated phosphoramidite oligonucleotide synthesis to afford the 3'-phosphate by established protocol.[46]

3. Base Content. It is realized that a high GC content increases the T_m of DNA duplexes.[47] The GC content in triplexes can also alter the T_m at the appropriate pH (where protonation of the Hoogsteen C is required). Examination of a double ligation between three homopurines where the central oligo fragment will be hexaguanine **11** (Scheme 4) will be compared to the same double ligation with central hexaadenine **10** (Scheme 3). This required the synthesis of templates **3** and **12**, which has already been completed. This will allow a direct comparison between A and G in ligation efficiencies.

Scheme 4.



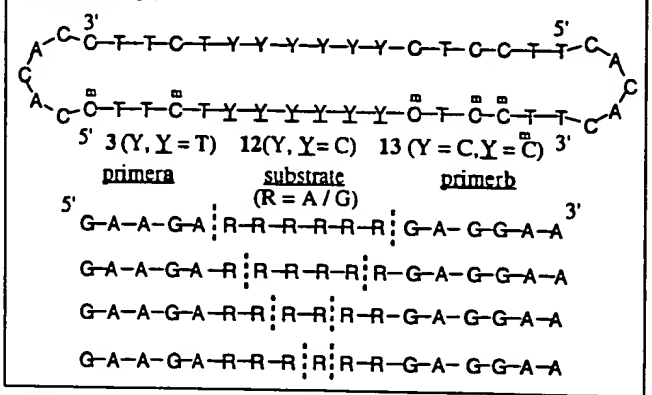
The C+GC triplet requires the Hoogsteen C to be protonated for two hydrogen bonds to be formed. This requirement establishes a handle for differentiating the two sides of the circular DNA template. The use of modified C derivatives on the Hoogsteen side of the circular template can either enhance their potential for protonation [as with 5-methylcytidine (^{Me}C)[35] can be replaced by modified derivatives which act as "permanently protonated" C derivatives [as with pseudoisocytidine (^{Pi}C)[36]]. Enforcing which side of the template will act as the Hoogsteen strand in the triplex complex by using modified C derivatives will allow regioselective control (3' vs. 5' directionality) of substrate binding to the template. This will minimize pyrophosphate formation. This factor will be assessed by comparing templates composed of all C (**12**) to all ^{Me}C derivatives (**13**) on the Hoogsteen side of hexaguanine **11** binding site of the circular template (Scheme 4). The ^{Me}C derivative should be protonated to a higher degree than the corresponding unmodified C.[35] Other ^{Pi}C template derivatives will be prepared [36] and studied as experiments dictate. These two templates will be compared to the hexaadenine binding template to assess their relative efficiencies for double ligation and conditions will be developed to minimize pyrophosphate formation.

4. Length of Ligating Fragments. The effect of the length of the ligating fragments are examined by performing the double ligation reaction with progressively longer primer segments (**a** and **b**, Scheme 5) along with a progressively shorter substrate. This will allow determination of the size limitation of the ligating segment.[39]

C. Product Turnover. Two primary approaches are examined to allow product turnover for repeated ligation cycles on the circular DNA. (1) A solution phase dialysis approach will first be examined. (2) If needed, a polyethyleneglycol (PEG) derivative may prove beneficial

for this solution-phase approach. It is anticipated that the solution phase dialysis approach will be amenable to scale-up and allow the puri-fication advantages of extractive washes.

Scheme 5.



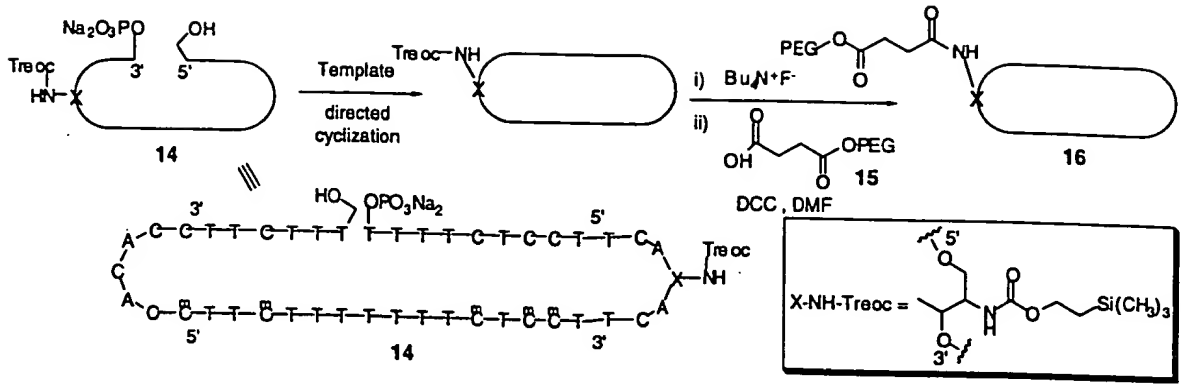
1. Dialysis Approach. The molecular weight (MW) of the smallest circular DNA template presently under study is approximately 18,000. This will be the lowest MW template used as all further template modifications will extensively increase the template size. The heptadecadeoxyribonucleotide product of ligation from this template has an approximate MW of 7,000. This MW difference is sufficient to allow separation with a 8,000 or 12,000 molecular weight cut off (MWCO) dialysis membrane. Advances in dialysis technology will allow the use of a microdialysis system with dialysis snap-capped microtubes to avoid sample loss through the tedious filling, tying and clamping of conventional dialysis tubing. Systems can be equipped for multiple sample capacity with oscillating and heating capabilities for efficient cyclical use.[48] This will allow development of a protocol for multiple cycles of template directed ODN synthesis in a reaction vessel to which capped dialysis reservoirs containing the circular template will be added. The substrates for template directed reactions can be added to the reaction vessel, equilibrated for template association, and the reaction initiated with addition of the activating reagent. After completion of the reaction the products can be separated through a simple denaturation and washing sequence. One cycle of oligonucleotide synthesis will consist of immersion of the dialysis reservoir containing the circular DNA template into a buffered reaction mixture containing the substrates to be ligated (or oligomerization monomers as the studies advance) along with $MgCl_2$. Template-substrate equilibrium will be established followed by addition of BrCN to initiate ligation. After ligation (<30 sec), the solution will be heated (for product denaturing), drained and washed (repeated as necessary). This cycle can be rapidly repeated (and readily automated) to produce the required amount of product. This approach will be discussed in more detail below (section II)

2. PEG-modified Circular Template. Should the dialysis approach require an increased molecular weight difference between the template and the ODN products for efficient separation, the circular template can be modified through a PEG attachment.[49] The biodegradable properties of this modification maintain the "green" aspects of this project.[50] Solution based synthesis of oligonucleotides by either the phosphotriester method or the phosphoramidite method has been optimized by using a soluble PEG support. PEG-modified oligonucleotides are well documented, making this modification quite routine.

Synthesis of the PEG-modified circular DNA template could conceivably be accomplished through linear solution phase synthesis of a branched oligonucleotide on a PEG support followed by triplex directed circularization. However, difficulty in purification of the PEG-modified circular DNA product might make this approach less feasible. Postsynthetic modification of a circular DNA template with a PEG attachment through a non-nucleotide branch point in the template will allow higher product purity. This can be accomplished by the following approach (Scheme 6). Conventional automated, phosphoramidite chemistry with the inclusion of a non-nucleotide phosphoramidite having a Treoc-protected amino group for functionalization will allow the synthesis of the required precircularized template.[51] Standard DMT-ON deprotection and cleavage from the solid support followed by purification with conventional RP-HPLC, removal of the DMT and a second RP-HPLC, will afford pure amino-protected, functionalized linear template **14** (Scheme 6). Triplex directed circularization by standard means[23d] followed by amino deprotection and PEG attachment through amidation with **15** will afford PEG-modified circular template **16** (Scheme 6). Any underivatized circular template can be removed through dialysis.

D. Multiple Ligations. Conditions derived from the previous double ligations are applied to triple ligations of trimers, quadruple ligations of dimers, and finally oligomerizations of monomers on circular DNA templates **3**, **12** and **13** (Scheme 7). Optimization is expected to

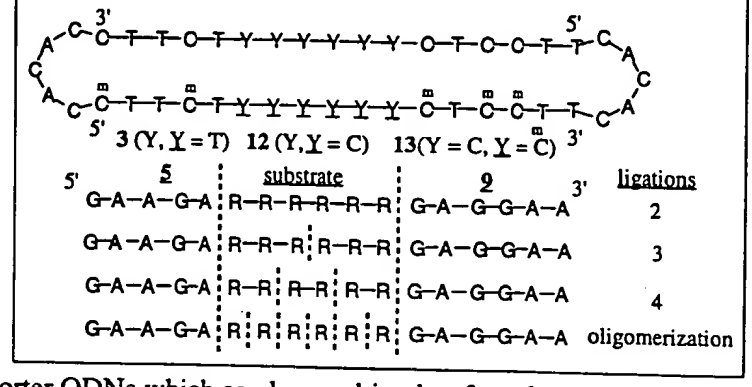
Scheme 6.



focus on the pre-equilibrium protocol, ionic strength, variation of substrate concentration, and the following modifications (section I.B).

1. Larger Circular DNA Templates. The length of the primer is expected to have an effect on the efficiency of these ligation reactions. The investigation of longer primers will require larger circular templates. Larger circular templates can be synthesized using the double ligation method on a triplex template as described by Kool and coworkers (Scheme 8).[23b] This will allow the synthesis and purification of shorter ODNs which can be combined to form larger and more complex template systems which will be described.

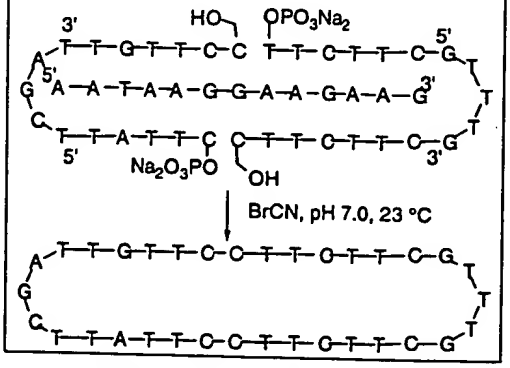
Scheme 7.



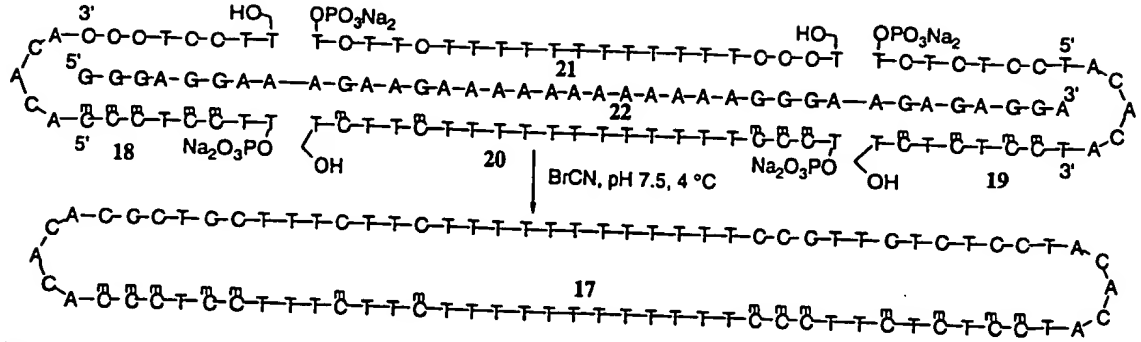
As an alternative to Kool's method, we will explore the potential of a quadruple ligation directed by a homopurine triplex template for the synthesis of the larger circular DNA template **17** (Scheme 9).[23b] The advantage of this quadruple ligation approach for circularization is the flexibility it will allow for the synthesis of a variety of circular DNA templates with differing sequences in the substrate binding regions of the template while maintaining constant primers. This approach will greatly enhance the efficiency of later studies for optimizing the use of circular DNA templates for oligonucleotide synthesis.

For the purpose of template design in this triplex directed quadruple ligation to form circular DNA, we have collaborated with Prof. Mark Arnold, a mathematician at the University of Arkansas, to develop a computer program which allows the rapid assessment of various sequences.[52] The purpose of this program is to optimize the sequence of the primer and substrate binding regions of the circular template so that undesired hybridizations are minimized. This has been used to design circular template **17** (Scheme 9), which will be synthesized through a triplex directed quadruple ligation reaction from the four oligonucleotides (**18-21**) on homopurine triplex template **22**. Regioselective alignment of **18-21** on template **22** will be achieved by incorporation of ^{Mc}C into the Hoogsteen side of oligonucleotides **18-20**. Alternatively, ^{pi}C will be incorporated as a "permanently protonated" C derivative. The combination of this regioselective control element, the requirement for 3'-OH to 5'-phosphate adjunction for ligation to occur, and the thermodynamic favorability for contiguous stacking when the four oligonucleotides are

Scheme 8.



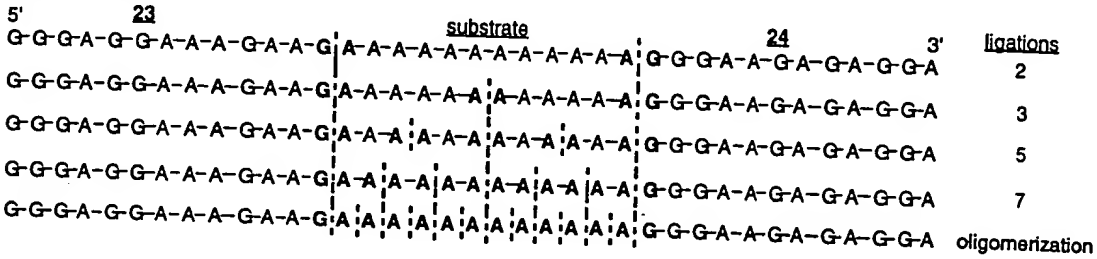
Scheme 9.



homogeneous in 3' to 5' directional alignment on triplex template **22** should favor desired **17**.

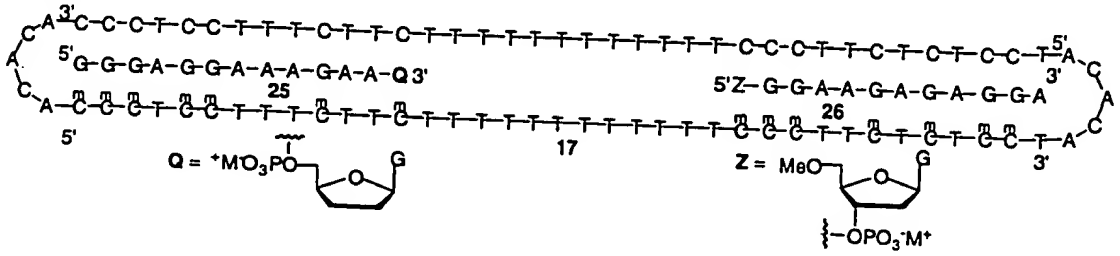
2. Longer Primers. For further studies of multiple ligations of shorter fragments and polymerization of monodeoxyribonucleotides, longer primers are examined to improve the cooperative binding strength in substrate/primer binding to the circular template. Circular template **17** (Scheme 9) will allow the use of a 5'-13mer primer **23** and a 3'-12mer primer **24** to be investigated (Scheme 10). This contains a intervening 12-mer substrate binding region. Oligomerization of adenosine (or uridine) has proved most challenging in nonenzymatic single strand template directed reactions.[53] Therefore, homoadenosine ligations and oligomerizations will be optimized for analysis of this template system. Progression from a double ligation between primers **23** and **24** and the homoadenosine 12-mer substrate up to the oligomerization between **23** and **24** and twelve 5'-adenosine mononucleotides will be examined.

Scheme 10.



3. Non-incorporated Primers. The present invention is also directed to primer modification to prevent their covalent reaction with the substrates so that the primers will not be ligated with the final ODN products. The primers will allow for template preorganization, cooperative binding benefits, and regiocontrol through contiguous stacking with the template substrates, but will not be covalently incorporated into the final product. This will require 3'- and 5'-end capping of primers **25** and **26**, respectively (Scheme **11**). This will be readily accomplished by synthesizing primer **25** with a 2',3'-dideoxy termination and primer **26** with a 5'-O-methyl termination. Primer **25** will be made on a DNA synthesizer using commercially available phosphoramidites for a reverse synthesis (5' to 3' direction) with the incorporation of the commercially available 2',3'-dideoxyguanosine phosphoramidite[54] for the final coupling. Primer **26** will require the synthesis of the 5'-O-methylguanosine phosphoramidite according to reported procedures.[55] Incorporation of this phosphoramidite as the final coupling on the DNA synthesizer will afford primer **26**. End-capped primers **25** and **26** will be used for template directed multiple ligations and oligomerizations as previously described (section I.D.2). The effect of the primers

Scheme 11.



on template directed reaction efficiency will be assessed by direct comparison to the reactions with the corresponding incorporated primers described above.

4. Primer-Circular Template Association. Further advancement towards a reusable or "catalytic" circular template to allow product turnover is examined by designing a circular template-primer system which will remain intact while allowing the products from the template directed substrate reaction to be separated using the dialysis approach briefly described (section I.C, see section II). This will require a method to bind the end-capped primers described above (section I.D.3) to the template while allowing the hybridization of the substrate with the template to be denatured. A covalent bound, end-capped primer attached to the circular template through an appropriate linker will allow multiple template use without primer separation.

A stable primer-template complex will be formed through covalent linkage of the end-capped primers to the circular DNA template. Extensive molecular modeling has been performed to optimize design of the template and primer.[56,57] Based on Kool's detailed analysis of optimal polyethyleneglycol linkers for the looped region of circular DNA for homopurine triplex binding, a modified polyethyleneglycol linker was chosen for modeling.[58] The biodegradable properties of this linker make it ideal for use in this "green process".[51] Kool's thorough studies found that a polyethyleneglycol linker between 23-30 Å was optimal for the looped region of circular DNA for triplex binding of single strand homopurine ODNs. For the modeling studies a linker composed of

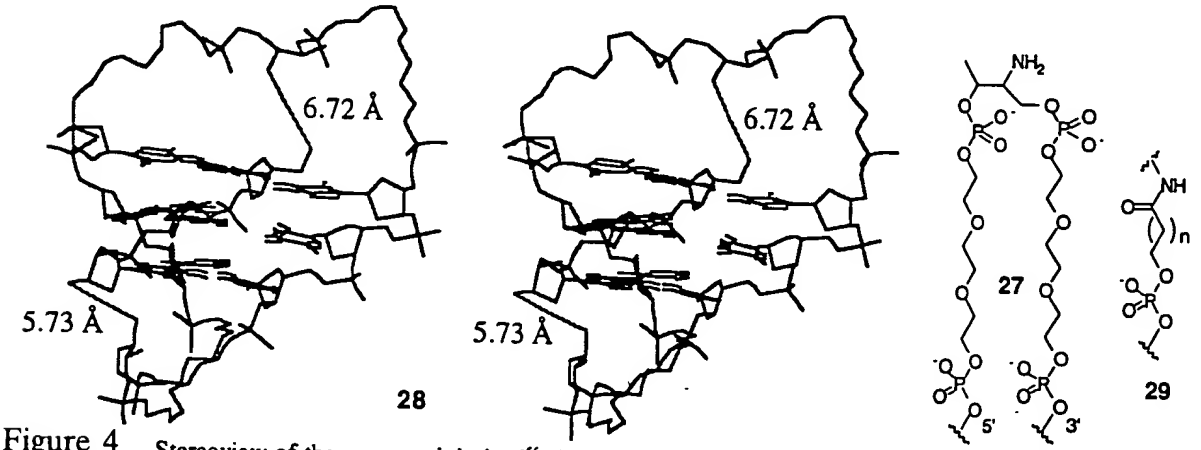


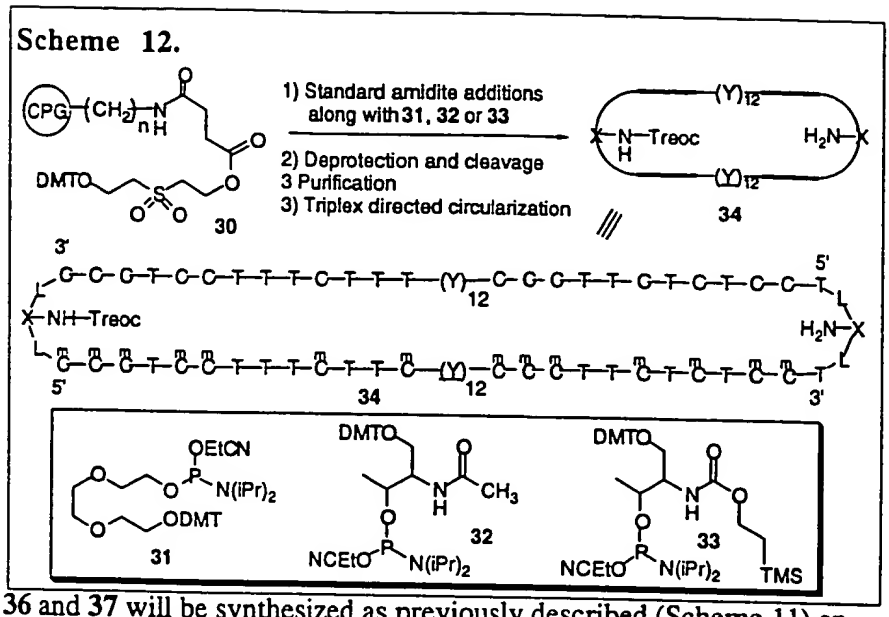
Figure 4. Stereoview of the energy minimized[56] circular DNA template (**28**) with looped linker **27** attached at both ends of the truncated triplex. Based on structure **28**, the initial template-primer linker for investigation will be **29** (n = 2). Based on an MM2 minimized structure, **29** will span a distance up to 7 Å.

two trikisethyleneglycol[56] units with an intervening threonine derived [59] unit connected through phosphodiester linkages was chosen (**27**, Fig. 4). The threonine unit was chosen based on its reported use as a functionalized linker. The stereocenter is expected to have a negligible effect due to the flexibility of the loop and linker. Energy minimization of a truncated model for the circular DNA template afforded structure **28** (stereoview, Fig. 4).[56] The distance between the amino group of the threonine derived linker and the 5'-oxygen of the central purine strand of the triplex was determined to be 6.72 Å. Similarly, on the 3'-end, the distance was 5.73 Å (Fig.4). Based on these modeling studies, Linker **29** (n = 1) was chosen to initiate the studies. An MM2 minimization of this linker suggested a span up to 7 Å. Incorporation of this linker will be described below. It is available in various lengths so a range of linkers can be assessed.

A convergent synthetic approach will be used to afford the fully functionalized circular template (Scheme 12). Again, this approach can be accomplished using standard, automated phosphoramidite chemistry. Oligonucleotide synthesis on succinyl-linked solid support **30** will be carried out with the insertion of commercially available trikisethyleneglycol phosphoramidite **31**[54] followed by threonine derived phosphoramidite **32**[59] and a second **31** for the first looped region. Additional oligonucleotide will be extended followed by the second loop region composed of **31**, Treoc-protected **33**, and another **31**. The template will be completed with the final oligonucleotide portion extension, DMT-ON deprotection and solid support linker cleavage and purification by RP-HPLC. After DMT removal and RP-HPLC purification, the linear modified oligo will be

circularized via triplex directed ligation with standard conditions to afford **34**.

Independently, the two modified primers will be synthesized (Fig.5). A carboxy terminus will be provided by using the nitroveratryl derivatized solid support **35** which can be photolytically cleaved to afford a carboxylic acid.[60] This derivatized support can be purchased in various linker lengths. Primer **36** and **37** will be synthesized as previously described (Scheme 11) on support **35**. The final circular DNA template **38** with covalently attached primers (Fig. 6) will be synthesized in two steps.[61] Treoc-protected **34** will be condensed with **37** and EDCI. Treatment with TBAF to remove the Treoc-protecting group followed by EDCI promoted condensation with **36** will afford the fully functionalized template **38** (Fig. 6).



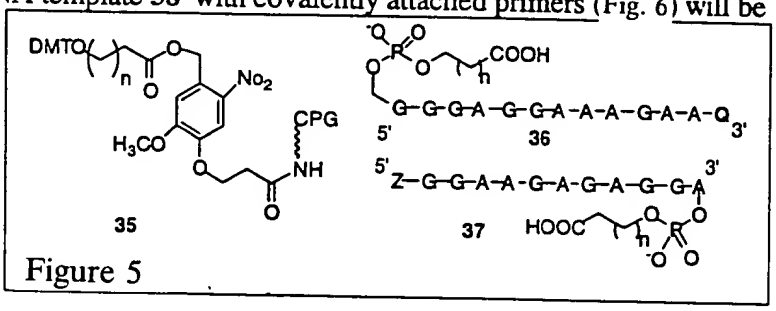


Figure 5

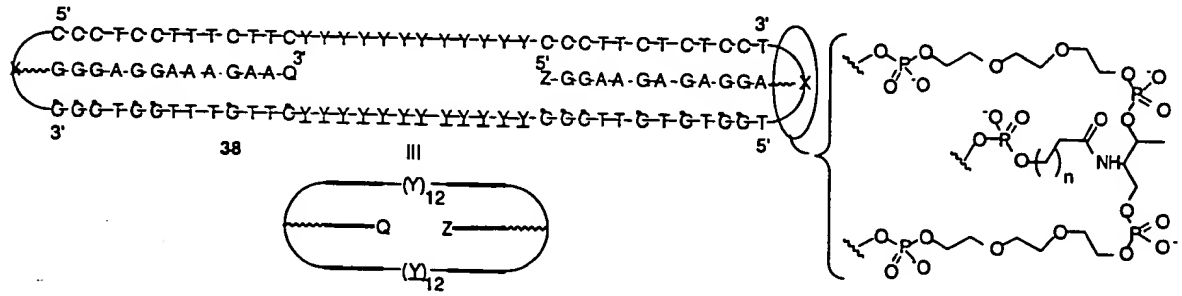


Figure 6 . Primer attached circular DNA template. See Scheme 11 for Q and Z designation.

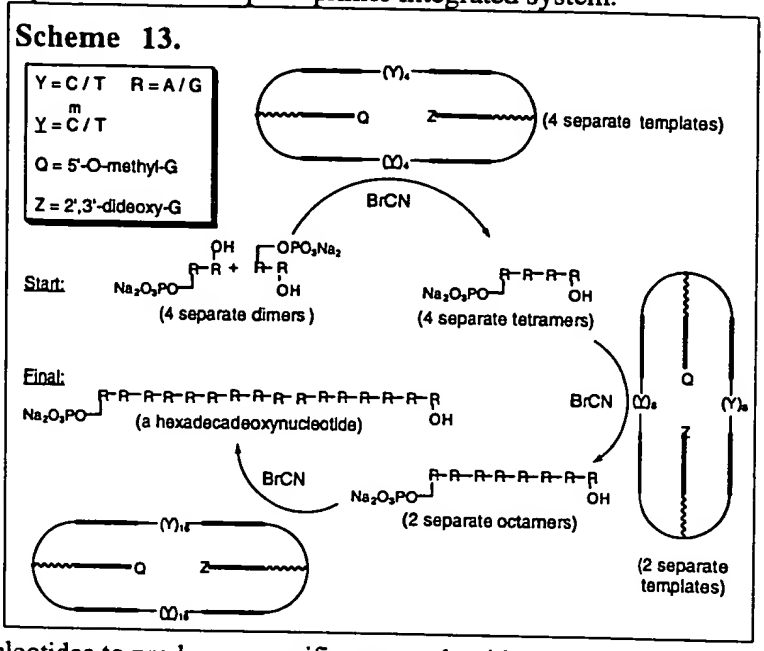
5. System Analysis. A combination of T_m, CD spectroscopy, and differential scanning calorimetry (DSC) will be used to derive as detailed an understanding of the template substrate association as possible. Analysis of the thermodynamics of mononucleotide association with the template system should be possible using DSC. These studies will be conducted in conjunction with Professor Wes Stites (University of Arkansas) whose expertise is in the area of protein dynamics study using DSC. Analysis of the enthalpic and entropic components of the substrate-template association will be particularly informative. Throughout the course of this project, comparison of various modifications in the template system, with appropriate controls not having the modification, will allow a detailed understanding of the effect of various template modifications (*e.g.*, ^{Me}C or ^{Pi}C incorporation, primer G-content, substrate binding region G-content, primer length, end capped primers, attached primers, primer-template linker length, length of PEG-looped region in the circular template).

II. Sequential Single Ligation Reactions.

Based on the quantitative ligation yields in our Preliminary Results with single ligation reactions on a circular DNA template, an additional option to a multiple ligation or oligomerization synthesis of ODNs will be pursued. This approach will utilize seven circular DNA templates with attached primers in seven separate template directed single ligation reactions to afford a homopurine hexadecanucleotide of any designed sequence. This will utilize all the previous synthetic approaches discussed to produce the required circular template-primer integrated system.

The basic approach is outlined in Scheme 13. The starting material source for template directed, single ligation reactions will be all four purine dideoxyribonucleotide monophosphates (pdAdA, pdAdG, pdGdA and pdGdG). These will be synthesized in large scale solution reactions by established methods.[62] The first template directed reaction will be a single ligation of two purine dinucleotides to afford one desired tetranucleotide. The second reaction with another two dinucleotides will form a second tetranucleotide. Repetition of these two reactions (third and fourth reactions) with two other pairs of dinucleotides will afford another two tetranucleotides. The fifth reaction will ligate two of the tetranucleotides to produce a specific octanucleotide while a sixth ligation reaction of another two tetranucleotides will give the second required octanucleotide for the final ligation reaction. The final, seventh ligation reaction between the two desired octanucleotides will afford the final hexadecanucleotide sequence specifically.

Scheme 13.



The process engineering aspects of large scale ODN synthesis using this technique could be accomplished in numerous ways. Two approaches will be investigated for ODN synthesis and purification. (1) The first, and most economic approach, will attempt the sequential ligation reactions in a single reaction vessel with no purification in a type of "in vitro selection process". Speed and simplicity will be the primary advantages of this approach. (2) The second approach will involve sequential dialysis of each ligation reaction and separate reaction vessels for each consecutive ligation. High purity and reaction efficiency will be the key advantages of this second approach.

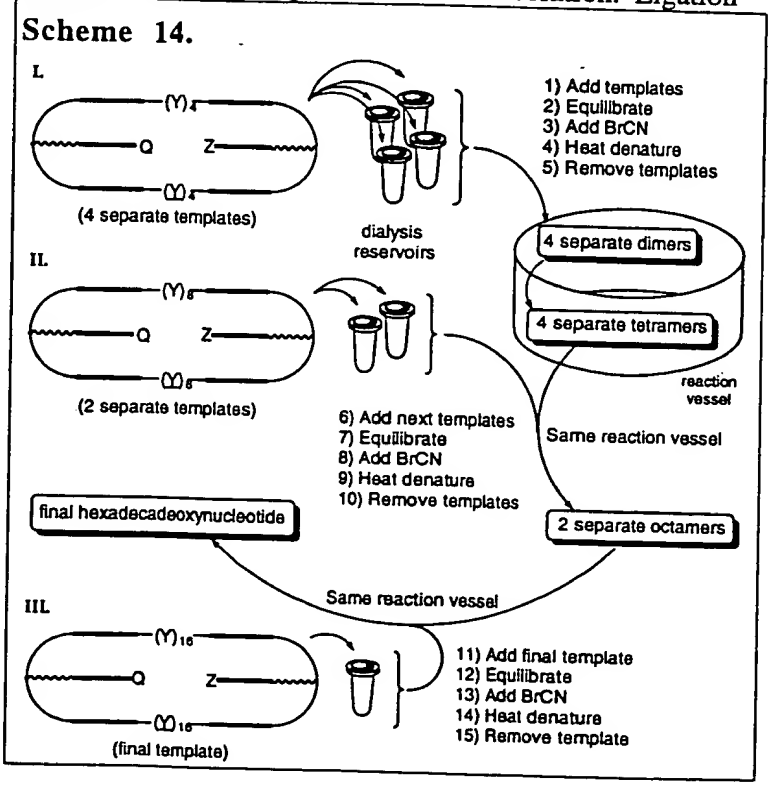
A. In vitro Selective Ligations. This economically efficient approach will require only one reaction vessel for the synthesis of a given homopurine hexadecadeoxyribonucleotide. This will be accomplished by having a reaction vessel containing all the required dinucleotides for ligation reactions to produce the four required tetranucleotides. Four separate MWCO 2,000 dialysis reservoirs, each containing a circular template designed for ligation of one of the tetramers, will be added to this reaction vessel. These dialysis reservoirs will allow the dinucleotide substrates to diffuse into the dialysis reservoirs and the tetramer ligation products of MW ~ 1,600 to diffuse out to the reaction vessel. A heating and cooling equilibration cycle (to be optimized) will allow template-substrate association. The low template association of mismatched dinucleotides and regioselective control imposed by primers and ^{Me}C or ^{pi}C incorporation in the template is expected to afford specificity in the template directed ligations. The ligation reaction will be initiated with the addition of BrCN. After a brief reaction time (<1 min, to be optimized) the four dialysis reservoirs will be removed, briefly washed, and any recovered tetramers added back to the reaction vessel. The dialysis reservoir containing the circular templates can be reused in separate reactions multiple times to afford the required amount of ODN. The two circular templates required for tetranucleotide ligations to afford octanucleotides will be added in MWCO 3,500

dialysis reservoirs to the reaction vessel. These dialysis reservoirs will allow diffusion of the substrate tetranucleotides in and diffusion of the octanucleotide ligation products (MW ~ 3,200) back into the reaction vessel. The same template-substrate equilibration, BrCN ligation initiation, and brief wash will afford the two desired octanucleotides in the single reaction vessel. Lastly, to this reaction vessel a dialysis reservoir (MWCO 8,000) containing the final template for octanucleotide ligation to afford the desired hexadecadeoxynucleotide will be added. The same sequence as before will afford a reaction mixture which should be highly concentrated with the final 16-mer. The buffered reaction conditions will prevent significant pH changes as BrCN decomposition products build up over the course of the reactions. The dialysis reservoirs containing the circular templates can be reused as necessary to produce the required amount of ODN. The final reaction mixture will be concentrated in a MWCO 3,500 dialysis reservoir to allow concentration of the final 16-mer (MW ~ 6,400) from any shorter ODNs in the solution (where the octamer will have a MW ~ 3,200). Any further purification, if required, can be accomplished by standard RP-HPLC or PAGE.

This will allow a type of "in vitro selection process" for thermodynamic selection of the most favored template-substrate association in each step. If mismatched ligations occur at one stage, the products will associate less tightly with the template for the following ligation. Excess ODN substrates from previous ligations should similarly not cause any complications since the longer ODN ligation products will always associate more tightly with the circular template. The higher template association during ligation pre-equilibrium will favor single ligation reactions of longer ODNs to afford the intended product ODN. The final dialysis will separate any starting substrates and truncated byproducts. This is anticipated to afford a superior, economical "green process" for homopurine ODN synthesis for large scale therapeutic or diagnostic applications.

B. Sequential Dialysis Ligations. The second approach will afford higher product purity from each ligation reaction, but require more time. This approach will involve sequential dialysis of each ligation reaction. Multiple reaction vessels will be used, and a series of circular templates with attached primers in dialysis reservoirs will be added (Scheme 14). The initial ligation of two dinucleotides to afford the desired tetranucleotides will be accomplished by adding the template (step I) in a MWCO 1,000 dialysis reservoir to a solution of the two required dinucleotides (MW ~ 800) followed by template-substrate equilibration for association. Ligation will then be initiated with the addition of BrCN. After < 1 min (conditions will be optimized), the dialysis reservoir with the product tetranucleotide (MW ~ 1,600) concentrated in the reservoir will be removed, and transferred to a MWCO 2,000 dialysis reservoir. Heat denaturing dialysis will afford the pure tetranucleotide. The circular template can then be transferred back to the MWCO 1,000 dialysis reservoir for repeated use. This cycle can be repeated as necessary to produce the required amount of ligation product. The other three tetranucleotides will be formed simultaneously in separate reaction vessels following the same procedure. The two desired tetranucleotides for the following octanucleotide synthesis will be combined in a reaction vessel to which the required template for the ligation reaction will be added in a

Scheme 14.



MWCO 2,000 dialysis reservoir (Step II, Scheme 14). Equilibration followed by ligation initiation with BrCN will afford the product octanucleotide (MW ~ 3,200) concentrated in the dialysis reservoir. As before, heat denaturing dialysis from a MWCO 8,000 reservoir will afford the pure octanucleotide. Again the remaining template can be reused for multiple ligation cycles to afford the desired amount of product. The same procedure will be used to produce the additional required octanucleotide simultaneously. Following the same cycle, the two octanucleotides will be placed in reaction vessel to which a MWCO 3,500 dialysis reservoir containing the final circular template will be added (Step III, Scheme 14). Equilibration, BrCN initiated ligation, and denaturing dialysis from a MWCO 8,000 reservoir will afford the pure hexadecadeoxyribonucleotide. Repeated cycles will afford as much product as required. This approach will afford pure 16-mer homopurine ODNs at potentially any desired scale. The yield of each ligation is expected to be high, as is the final product purity since all precursors and byproducts are removed at each stage of the sequential ligations.

C. Methodology Advantages. There are six important advantages to these approaches. (1) The "catalytic" use of the templates will allow multiple cycles of each ligation reaction to be performed to produce large quantities of each oligonucleotide. (2) The high yield of each single ligation reaction (based on quantitative yields seen in Preliminary Results) should allow high throughput and efficiency. (3) This dialysis based approach should afford ODNs of high purity and allow the circular templates to be efficiently reused with little or no loss of the template. (3) The use of unprotected nucleotides, cyanogen bromide and magnesium chloride in buffered water allows for a highly economic approach for the synthesis of defined oligonucleotides.[7] (4) The fast rate of these ligation reactions also enhances the economics of this methodology through short cycle times. (5) This will afford an optimal "green process". The byproducts and waste from this synthetic approach are harmless to the environment. All unreacted ODNs can be recycled to minimize the loss of any starting materials. (6) The combinatorialization of this methodology can allow the synthesis of all possible homopurine hexadecadeoxyribonucleotides. The combination of all these advantages should make this methodology for homopurine ODN synthesis an extremely attractive approach in regard to production scale, efficiency, cost,[7] and environmental impact.

III. Future Studies.

The results of these proposed investigations will clearly define the potential of this template directed methodology for ODN synthesis. Future studies will span over many areas. Modification of the template backbone with nucleic acid derivatives such as peptide nucleic acids (PNA)[63] will allow variation in template-substrate association for expansion of the types of substrates and reactions which can be directed by the template.[64] Non-nucleic acid pyrimidine- and purine-like bases[65] will be incorporated in order to expand the pool of substrates for template binding. This will allow the synthesis of modified nucleic acids for improved biodelivery and biostability properties for diagnostic and therapeutic applications. This will also lead to the synthesis of a variety of functionally controlled oligomers of all types. A variety of monomers with cleavable nucleobase components attached for template association will allow limitless oligomerizations to be directed by the sequence defined template. The nucleobase component can then be cleaved to release the template directed oligomer which was synthesized. This would be a "template auxiliary" approach to controlled oligomerizations. Several of these aspects are already being pursued in separate projects in our laboratories. In total, the basic methodology is anticipated to allow a variety of unique synthetic reactions to be studied and interesting oligomers and functional materials to be produced.

References Cited

[1] Hileman.B. *C&EN* (June 8) **1998**, 31. http://www.lanl.gov/Internal/projects/green.

[2] Niedle, S. "Recent Developments in Triple-Helix Regulation of Gene Expression," *Anti-Cancer Drug Design* **1997**, *5*, 433-42.

[3] (a) Christoffersen, R.E. "Translating Genomics Information into Therapeutics: A Key Role for Oligonucleotides," *Nature Biotech.* **1997**, *15*, 483-84. (b) Alama, A.; Barbieri, F.; Cagnoli, M.; Schettini, G. "Antisense Oligonucleotides as Therapeutic Agents," *Pharm.Res.* **1997**, *36*, 171-78. (c) Temsamani, J.; Guinot, P. "Antisense Oligonucleotides: A New Therapeutic Approach,"*Biotech. and Appl. Biochem.* **1997**, *26*, 65-71. (d) Maher, L.J. "Prospects for the Therapeutic use of Antigene Oligonucleotides," *Cancer Investig.* **1996**, *14*, 66-82. (e) Crooke, S.T.; Bennett, C.F. "Progress in Antisense Oligonucleotide Therapeutic," *Ann.Rev. Pharm. Toxic.* **1996**, *36*, 107-29. (f) Dean, N.M.; Mckay, R.; Miraglia, L.; Geigert, T.; Müller, M.; Fabbro, D.; Bennett, C.F. "Antisense Oligonucleotides as Inhibitors of Signal Transduction-Development From Research Tools To Therapeutic Agents," *Biochem.Soc.* Trans. **1996**, *24*, 623-29. (g) Rojanasakul, Y.Y. "Antisense Oligonucleotide Therapeutics - Drug Delivery and Targeting," *Adv. Drug Del.Rev.* 1996, *15*, 115-31. (h) Demesmaeker, A.D.; Häner, R.; Martin, P.; Moser, H.E. "Antisense Oligonucleotides," *Acc. Chem. Res.* **1995**, *28*, 366-74.

[4] (a) Pastinen, T.; Kurg, A.; Metspalu, A.; Peltonen, L. "Minisequencing: A Specific Tool for DNA Analysis and Diagnostics on Oligonucleotide Arrays," *Genome Res.* **1997**, *7*, 606-14. (b) Yershov, G.; Barsky.; Belgovskiy, A.; Kirrillov, E.; Kriendlin, E.; Ivanov, I.; Parinov, S.; Guschin, D.; Drobishev, A.; Dubiley, S.; Mirzabekov, A. "DNA Analysis and Diagnostics on Oligonucleotide Microchips," *Proc. Natl. Acad. Sci. USA 1996*, *93*, 4913-18. (c) Gold, L. "Oligonucleotides as Research, Diagnostic, and Therapeutic Agents," *J.Biol. Chem.* **1995**, *270*, 13581-84. (d) Assman, G. "Research in Gene Diagnostics of Cancer Disease," *Gene* **1995**, *159*, 9-10. (e) Fahy, E.; Davis, G.R.; DiMichele, L.J.; Ghosh, S.S. "Design and Synthesis of Polyacrylamide-Based Oligonucleotide Supports for Use in Nucleic Acid Diagnostics," *Nucleic Acids Res.* **1993**, *21*, 1819-26 (f) Cotton, R.G.H. "Current Methods of Mutation Detection," *Mutat. Res.* **1993**, *285*, 125-144.

[5] For examples of Oligonucleotides for Molecular Biology applications see: (a)Marshall, W.S.; Boymel, J.L. "Oligonucleotide Synthesis as a Tool in Drug Discovery Research," *Drug Discovery Today* **1998**, *3*, 34-42. (b) Potapov, V.K.; Azhikina, T.L..; Demin, V.V.; Sverdlov, E.D. " Modified Oligonucleotides as a Tool for DNA Sequencing, Fingerprinting and Mapping," *Pure & Appl. Chem.* **1996**, *68*, 1315-20 (c) Pfleiderer, W.; Matysiak, S.; Bergmann, F.; Schnell, R. "Recent Progress in Oligonucleotide Synthesis," *Acta Biochimica Polonica* **1996**, 477-487. (d) Huynhdinh, T. "Modified Synthetic Oligonucleotides as Tools for Molecular Biology," *Bulletin De L Institut Pastuer* **1990**, 139-157

[6] Murphy, M.; Reiger, M.; Jayaraman, K.; "Large-Scale Synthesis of Triple Helix Forming Oligonucleotides Using a Controlled Pore Glass Support," *Biotechniques* **1993**, *15*, 1004-07.

[7] The cost difference was determined according to present catalog prices as follows:

Regular Approach

Reagents	Amount/Base	Cost dollars/Unit	Dollars/Base
Amidities	0.02 g	$40/ g	$0.80
Activator	0.15 ml	$1.00/ ml	$0.15
Capping solution A	0.2 ml	$0.1388/ ml	$0.02926
Capping solution B	0.22 ml	$0.33/ml	$0.0726
Oxidizer	0.44 ml	$0.155/ml	$0.0651
Deblock Solution	0.55 ml	$0.088/ml	$0.0484

Acetonitrile	15 ml	$0.083/ml	$1.25
		Total	**$2.50**

Costs are based on 0.2 μM synthesis. In addition to the reagent costs, one time cost for solid support, machine and hazardous material shipment charges.

Our Approach

Reagents	Amount/Base	Cost dollars/Unit	Dollars/Base
G-mono phosphate	0.0001g	$3.92/ g	$0.000392
A-mono phosphate	0.0001 g	$1.89/ g	$0.000189
BrCN (5.0 M) in CH_3CN	2 μL	$3.86/ ml	$0.000772
		Total	**$0.001353**

Costs are based on 0.2 μM synthesis. In addition to the reagent costs, one time cost for template synthesis.

[8] (a) Egli, M. "Structural Aspects of Nucleic Acid Analogs *and Antisense Oligonucleotides,"Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 1894-1909. (b) Beaucage, S.L.; Iyer, R.P. "The Synthesis of Modified Oligonucleotides by the Phosphoramidite Approach and their Applications," *Tetrahedron* **1993**, 6123-6194. (c) Verma, R.S. "Synthesis of oligonucleotide analogues with modified backbones," *Synlett* **1993**, 621-637. (d) Englisch, U.; Gauss, D.H. "Chemically Oligonucleotides as Probes and Inhibitors," *Angew. Chem. Int. Ed. Engl.* **1991**, *30*, 613-629. (e) Uhlmann, E.; Peyman, A. " Antisense Oligonucleotides - A New Therapeutic Principle,"*Chem. Rev.* **1990**, *90*, 543-84.

[9] For a complete resource covering many aspects of the human genome see the Human Genome Database (GDB) hosted at Johns Hopkins University (http://www.gdb.org/): Letovsky, S. I.; Cottingham, R.W.; Porter, C.J.; Li, P.W.D. "GDB: The Human Genome Database." *Nucleic Acids. Res.* **1998**, *26*, 94-99.

[10] For a selection of recent examples, see: (a) Matteucci, M.; Lin, K.-Y.; Huang, T.; Wagner, R.; Sternbach, D.D.; Mehrotra, M.; Besterman, J.M. "Sequence-Specific Targeting of Duplex DNA Using a Camptothecin-Triple Helix Forming Conjugate and Topoismerase I," *J. Am. Chem. Soc.* **1997**, *119*, 6939-40. (b) Faruqi, A.F.; Seidman, M.M.; Segal, D.J.; Carroll, D.; Glazer, P.M. "Recombination Induced by Triple-Helix-Targated DNA Damage in Mammalian Cells," *Mol. and Cell. Biol.* **1996**, *16*, 6820-28. (c) Bouazine, M.; Cherny, D.I.; Mouscadet, J.-F.; Auclair, C. "Alternate Strand DNA Triple Helix-Mediated Inhibition of HIV-1 U5 Long Terminal Repeat Integration in Vitro," *J. Biol. Chem.* **1996**, *271*, 10359-64.

[11] (a) Beaucage, S.L.; Caruthers, M.H. "Deoxynucleoside Phosphoramidites. A New Class of Key Intermediates for Deoxypolynucleotide Synthesis," *Tetrahedron Lett.* **1981**, 22, 1859-62. (b) Matteuci, M.D.; Caruthers, M.H. "Synthesis of Deoxyoligonucleotides on a Polymer Support," *J. Am. Chem. Soc.* **1981**, *103*, 3185-91. (c) Beaucage, S.L. and Iyer, R.P. "Advances in the Synthesis of Oligonucleotides by the Phosphoramidite Approach," *Tetrahedron* **1992**, *48*, 2223-31.

[12] (a) Reese, C.B. "The Chemical Synthesis of Oligo- and Poly-Nucleotides by the Phospho Triester Approach," *Tetrahedron* **1978**, *34*, 3143-79. (b) Narang, S.A. "DNA Synthesis," *Tetrahedron* **1983**, *39*, 3-22. (c) Itakura, K.; Rossi, J.J.; Wallace, R.B. *Annual. Rev. Biochem.* **1984**, *53*, 323-56. (d) Effimov, V.A.; Burayakova, A.A.; Dubey, I.Y.; Polushin, N.N.; Chakhmakhcheva, O.G.; Ovchinnikev, Yu.A. "Applications of new Catalytic Phosphate Protecting groups for the Highly Efficient Phosphodiester Oligonucleotide Synthesis," *Nucleic Acids Res.* **1986**, *14*, 6525-40. (d) Reese, C.B.; Zhang, P.-Z. "Phosphotriester approach to the Synthesis of Oligonucleotides: A Reappraisal," *J.Chem. Soc. Perkin Trans 1* **1993**, 2291-301.(e)

Reese, C.B.; Song, Q.L. "A New Appraoch to the Synthesis of Oligonucleotides and their Phosphorthioate analogues in solution," *Bioorg. & Med. Chem. Lett.* **1997**, 2787-2792.

[13] (a) Froehler, B.C.; Matteucci, M.D. " Nucleoside-H Phosphonates: Valuable Intermediates in the Synthesis of Deoxynucleotides," *Tetrahedron Lett*, **1986**, *27*, 469-72. (b) Garegg, P.T.; Lindh, I.; Regberg, T.; Stawinski, T.; Stromberg, R. "Nucleoside H-Phosphonates IV. Automated Solid Phase Synthesis of Oligoribonucleoside by the Hydrogen Phosponate Approach," *Tetrahedron Lett.* **1986**, *27*, 4051-57. (c) Zaramella, S.; Bonora, G.M. "The Application of H-Phosphonate Chemistry in the Help Synthesis of Oligonucleotides," *Nucleosides and Nucleotides* **1995**, *14*, 809-12.

[14] Andrade, M.; Scozzari, A.S.; Cole, D.L.; Ravikumar, V. T. "Efforts Toward Synthesis of Oligonucleotides for Commercialization," *Nucleosides and Nucleotides* **1997**, 1617-20.

[15] (a) Bonora, G.M.; Scremin, C.L.; Colonia, F.P.; Garbesi, A. "HELP (High Efficiency Liquid Phase) New Oligonucleotides Synthesis On Soluble Polymeric Support," *Nucleic Acids Res.* **1990**, *18*, 3155-3159. (b) Gravert, D.J.; Janda, K.D. "Organic Synthesis on soluble polymer Supports: Liquid phase Methodologies," *Chem. Rev.* **1997**, *97*, 489-509.

[16] Porath, J. "From Gel Filtration to Adsorptive Size Exclusion," *J.Protein Chem.* **1997**, 463-466.

[17] Bonora, G.M. "Polyethylene glycol-A High Efficiency Liquid phase (HELP) for the Large-Scale synthesis of the Oligonculeotides," *Applied Biochemistry and Applied Biotechnology* **1995**, 3-17.

[18] (a) Hobbs, J.D. "Nucleotides and Nucleic Acids," in *Organophosphorous Chemistry* **1990**, *21*, 201-321. (b) Sheikh, S.N. Lazarus, P. "Re-usable DNA Template for the Polymerase Chain Reaction (PCR)," *Nucleic Acids Res.* **1997**, *25*, 3537-42.

[19] Naylor, R.; Gilham, P.T. "Studies on Some Interactions and reactions of Oligonucleotides in Aqueous Solution," *Biochemistry* **1966**, *5*, 2722-28.

[20] (a) Kanaya, E.; Yanagawa, H. "Template-Directed Polymerization of Oligoadenylates Using Cyanogen Bromide," *Biochemistry* **1986**, *25*, 7423-30. (b) Zuber, G.; Sirlin, C.; Behr, J.P. "Enhanced Ligation of DNA with a Effector Molecule," *J. Am. Chem. Soc.* **1993**, *115*, 4939-40. (c) Dolinnaya, N.G.; Sharbova, Z.A. "Chemical Ligation as a Method for the Assembly of Double-Stranded Nucleic Acids: Modifications and Local Structure Studies," *Russ. Chem. Bull.* **1996**, *45*, 1787-1809.

[21] (a) Luebke, K.J.; Dervan, P.B. "Nonenzymatic Ligation of Oligodeoxyribonucleotides on a Duplex DNA Template by Triple-Helix Formation," *J. Am. Chem. Soc.* **1989**, *111*, 8733-35. (b) Luebke, K.J.; Dervan, P.B. "Nonenzymatic Ligation of Double-Helical DNA by Alternate-Strand Triple Helix Formation," *Nucleic Acids Res.* **1992**, *20*, 3005-09. (c) Dolinnanya, N.G.; Pyatarauskene, O.V.; Shabarova, Z.A. "Probing DNA Triple Helix Structure by Chemical Ligation," *FEBS Lett.* **1991**, *284*, 232-34

[22] (a) Zeilinski, W.S.; Orgel, L.E. "Auto Catalytic Synthesis of a Tetra Oligonucleotide Analogue,"*Nature* **1987**, *327*, 346-47. (b) von Kiedrowski, G.; Wlotzka, B.; Helbing, J.; Matzen, M.; Jordan, S. "Parabolic Growth of a Self-Replicating Hexadeoxynucleotide Bearing a 3'-5'-Phosphoamidate Linkage," *Angew. Chem. Int. Ed. Engl.* **1991**, *30*, 423-26. (c) Dolinnaya, N.G.; Sokolova, N.I.; Ashirbekova, D.T.; Shabarova, Z.A. "*Nucleic Acids Res.* **1991**, *19*, 3067-72. (d) Rumney IV, S.; Kool, E.T. "DNA Recognition by Hybrid Oligoether-Oligodeoxynucleotide Macrocycles," *Angew. Chem. Int. Ed. Engl.* **1992**, *31*, 1617-19. (e) Luo, P.; Leitzel. J.C.; Zhan, Z.-Y.J.; Lynn, D.G. "Analysis of the Structure and Stability of a Backbone-Modified Oligonucleotide: Implications for Avoiding Product Inhibition in Catalytic

Template-Directed Synthesis," *J. Am. Chem. Soc.* **1998**, *120*, 3019-31. (f) Sawai, H.; Totsuka, S.; Yamamoto, K.; Ozaki, H. "Non-Enzymatic, Template-Directed Ligation of 2'-5' Oligoribonucleotides. Joining of a Template and a Ligator Strand," *Nucleic Acids Res.* **1998**, *26*, 2995-3000. (g) Herrlein, M.K.; Letsinger, R.L. "Selective Chemical Autoligation on a Double-Stranded DNA Template," *Nucleic Acids Res.* **1994**, *22*, 5076-78. (h) Bohler, C.; Nielsen, P.E.; Orgel, L.E. "Template Switching Between PNA and RNA Oligonucleotides." *Nature* **1995**, *376*, 578-81. (i) Pitsch, S.; Krishnamurthy, R.; Bolli, M.; Wenderborn.S.; Holzner, A.; Minton, M.; Lesueur, C.; Schlönvogt, I.; Jaun, B.; Eschenmoser, A. "Pyranosyl-RNA ('p-RNA'): Base-Pairing Selectivity and Potential to Replicate," *Helv. Chim. Acta.* **1995**, *78*, 1621-34.(j) Schmidt, J.G.: Christensen, L.; Nielsen, P.E.; Orgel, L.E. "Information Transfer from DNA to Peptide Nucleic Acids by Template-Directed Syntheses," *Nucleic Acids Res.* **1997**, *25*, 4792-96. (k) Bruick, R.K.; Dawson, P.E.; Kent, S.B.H.; Usman, N.; Joyce, G.F. "Template-Directed Ligation of Peptides to Oligonucleotides," *Chem. & Biol.* **1996**, *3*, 49-56. (l) Gryanzov, S.M.; Letsinger, R.L. "Chemical Ligation of Oligonucleotides in the Presence and Absence of a Template," *J. Am. Chem. Soc.* 1993, *115*, 3808-09.

[23] (a) Prakash, G.; Kool, E.T. "Structural Effects in the Recognition of DNA by Circular Oligonucleotides," *J. Am. Chem. Soc.* **1992**, *114*, 3523-27. (b) Rubin, E.; Rumney IV. S.; Wang, S.; Kool, E.T. "Convergent DNA Synthesis: A Non-Enzymatic Dimerization Approach to Circular Oligodeoxynucleotides," *Nucleic Acids Res.* **1995**, *23*, 3547-53. (c) Li, T.; Weinstein, D.S.; Nicolaou, K.C. "The Chemical End-Ligation of Homopyrimidine Oligodeoxyribo-nucleotides within a DNA Triple Helix," *Chem. & Biol.* **1997**, *4*, 209-14. (d) Dolinnaya, N.G.; Blumenfeld, M.; Merenkova, I.N.; Oretskaya, T.S.; Krynetskaya, N.F.; Ivanovskaya, M.G.; Vasseur, M.; Shabarova, Z.A. "Oligonucletoide Circularization by Template-Directed Chemical Ligation," *Nucleic Acids Res.* **1993**, *21*, 5403-07. (e) Herrlein, M.K.; Nelson, J.S.; Letsinger, R.L. "A Covalent Lock for Self-Assembled Oligonucleotide Conjugates," *J. Am. Chem. Soc.* **1995**, *117*, 10151-52. (f) Xu, Y.; Kool, E.T. "Chemical and Enzymatic Properties of Bridging 5'-S-Phosphorothioester Linkages in DNA," *Nucleic Acids Res.* **1998**, *26*, 3159-64.

[24] (a) Li, T.; Nicolaou, K.C. "Chemical Self-Replication of Palindromic Duplex DNA," *Nature* **1994**, *369*, 218-21. (b) Bag, B.G.; von Kiedrowski, G. "Templates. Autocatalysis and Molecular Replication," *Pure & Appl. Chem.* **1996**, *68*, 2145-52. (c) Rohatgi, R.; Bartel, D.P.; Szostak, J.W. "Kinetic and Mechanistic Analysis of Nonenzymatic Template-Directed Oligoribonucleotide Ligation," *J. Am. Chem. Soc.* **1996**, *118*, 3332-39. (d) Bolli, M.; Micura, R.; Eschenmoser, A. " Pyranosyl RNA: Further Observations On Replication," *Chem. & Biol.* **1997**, *4*, 309-20.

[25] (a) Wu, T.; Orgel, L.E. "Nonenzymatic Template-Directed Synthesis on Oligodeoxycytidylate Sequences in Hairpin Oligonucleotides," *J. Am .Chem. Soc.* **1992**, *114*, 317-22. (b) Wu, T.; Orgel, L.E. "Nonenzymatic Template-Directed Synthesis on Hairpin Oligonucleotides. 2. Templates Containing Cytidine and Guanosine Residues," *J. Am .Chem. Soc.* **1992**, *114*, 5496-501. (c) Wu, T.; Orgel, L.E. "Nonenzymatic Template-Directed Synthesis on Hairpin Oligonucleotides. 3. Incorporation of Adenosine and Uridine Residues," *J. Am .Chem. Soc.* **1992**, *114*, 7964-69.(d) Schmidt, J.G.; Nielsen, P.E.; Orgel, L.E. "Information Transfer from Peptide Nucleic Acids to RNA by Template-Directed Syntheses," *Nucleic Acids Res.* **1997**, *23*, 4797-802. (e)Rodriguez, L.; Orgel. L.E. "Template-Directed Extension of a Guanosine 5'-Phosphate Covalently Attached to an OligoDeoxycytidylate Template," *J.Mol. Evol.* **1991**, *33*, 477-82. (f) Rembold, H.; Orgel, L.E. "Single-Strand Regions of Poly (G) Act as Templates for Oligo (C) Synthesis, *J.Mol. Evol.* **1994**, *33*, 205-10. (g) Ertem, G.; Ferris, J.P. "Synthesis of RNA Oligomers on Heterogeneous Templates," *Nature* **1996**, *379*, 238-40. (g) Schwartz, A.W.; Orgel, L.E. "Template-Directed Synthesis of Novel, Nucleic Acid-Like Strucures," *Science* **1985**, *228*, 585-587. (h) Stribling, R.; Miller, S.L. "Template-Directed Synthesis of Oligonucleotides Under Eutectic Conditions, " *J.Mol. Evol.* **1991**, *32*, 289-95. (h) Kurz, M.; Göbel, K.; Hartel, C.; Göbel, M.W. "Nonenzymatic Oligomerization of Ribonucleotides on Guanosine-Rich Templates: Suppression of the Self-Pairing of Guanosine," *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 842-45. (I) Prakash, T.P.; Roberts, C.; Switzer, C. "Activity of 2'5'-Linked

RNA in the Template-Directed Oligomerization of Mononucleotides," *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 1522-23.

[26] (a) Kanavarioti, A. "Self-Replication of Chemical Systems Based on Recognition Within a Double or a triplex- A Realistic Hypothesis," *J. Theor. Biol.* **1992**, *158*, 207-19. (b) Kanavarioti, A. "Template-Directed Chemistry and the Origins of the RNA World," *Orig. Life Evol. Biosphere* **1994**, *24*, 479-94. (c) Inoue, T.; Orgel, L.E. " A Nonenzymatic RNA Polymerase Model," *Science* **1983**, *219*, 859-62. (d) Orgel, L.E.; Lohrmann, R. "Prebiotic Chemistry and Nucleic Acid Replication," *Acc. Chem. Res.* **1974**, *7*, 368-377.

[27] Joyce, G.F. "Nonenzymatic Template-Directed Synthesis of Informational Macromolecules," *Cold spring Harbor Symposium on Quantitative Biology*, Cold Spring Harbor, NY, **1987**, Vol. LII, pp 41-51.

[28] (a) Hill, Jr. A.R.; Dee Nord, L.; Orgel, L.E.; Robins, R.K. "Cyclization of Nucleotide Analogues as an Obstacle to Polymerization," *J. Mol. Evol.* **1988**, *28*, 170-71. (b) Kanavarioti, A. "Dimerization in Highly Concentrated Solutions of Phosphoimidazolide Activated Mononucleotides," *Orig. Life Evol. Biosphere* **1997**, *27*, 357-76.

[29] (a) Harada, K.; Orgel, L.E. "In Vitro Selection of Optimal DNA Substrates for Ligation by a Water-Soluble Carbodiimide," *J. Mol. Evol.* **1994**, *38*, 558-60. (b) James, K. D.; Ellington, A.D. "Surprising Fidelity of Template-Directed Chemical Ligation of Oligonucleotides," *Chem. & Biol.* **1997**, *4*, 595-605.

[30] (a) Goodwin, J.T.; Lynn, D.G. "Template-Directed Synthesis-use of a Reversible Reaction," *J. Am. Chem. Soc.* **1992**, *114*, 9,197-98. (b) Zhan, Z.Y.J.; Lynn, D.G. "Chemical Amplification Through Template-Directed Synthesis," *J. Am. Chem. Soc.* **1997**, *119*, 12420-21.

[31] (a) Liu, D.; Daubendiek, S.L.; Zillman, M.A.; Ryan, K.; Kool, E.T. "Rolling Circle DNA Synthesis: Small Circular Oligonucleotides as Efficient Templates for DNA Polymerases," *J. Am. Chem. Soc.* **1996**, *118*, 1587-94. (b) Daubendiek, S.L.; Kool, E.T. "Genertation of Catalytic RNAs by Rolling Transcription of Synthetic DNA Nanocircles," *Nature Biotech.* **1997**,*15*, 273-277.

[32] (a) Fire, A.; Xu, Si-Q. "Rolling Replication of Short DNA Circles," *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 4641-45.

[33] Kool, E.T.; "Recognition of DNA, RNA, and Proteins by Circular Oligonucleotides," *Acc.Chem. Res.* **1998**, *31*, 502-10. (b) Kool, E.T. "Design of Triplex-Forming Oligonucleotides for Binding DNA and RNA: Optimizing Affinity and Selectivity," *New. J. Chem.* **1997**, *21*, 33-45.(c) Kool, E.T. "Preorganization of DNA: Design Principles for Improving Nucleic Acid Recognition by Synthetic Oligonucleotides," *Chem. Rev.* **1997**, *97*, 1473-1487

[34] (a) Soyfer, V.N.; Potaman, V.N. "Triple Helical Nucleic Acids," Springer-Verlag: New-York, **1996**. (b) Sun, J.S.; Garestier, J.; Helene, C. "Oligonucleotide Directed triple Helix Formation," *Curr. Opinion in Structural Biol.* **1996**, *6*, 327-33. (c) Doronina, S.; Behr, J.-P. "Towards a General Triple Helix Mediated DNA Recognition," *Chem. Soc. Rev.* **1997**, 63-71

[35] (a) Plum, G.E.; Park, Y.-W.; Singleton, S.F.; Dervan, P.B.; Breslauer, K.J. *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 9436-9440. (b) Xodo, L.E.; Manzini, G.; Quadrifoglio, F.; Van der Marel, G.A.; van Boom, J.H. *Nucleic Acids Res.* **1991**, *19*, 5625-31. (c) Hunziker, J.; Priestley, E.S.; Brunar, H.; Dervan, P.B. *J. Am. Chem. Soc.* **1995**, *117*, 2661-62.

[36] (a) Ono, A.; Ts'O, P.O.P.; Kan, L.S. "Triplex Formation of an Oligomer Containing 2'-O-Methyl Pseudo IsoCytidine With a DNA Duplex at Neutral pH," *J.Org.Chem.* **1992**, *57*, 3225-30. (b) Ono, A.; Ts'O, P.O.P.; Kan, L.S. "Triplex Formation of an Oligomer Containing

2'-O-Methyl Pseudo IsoCytidine With Single and Double Stranded Nucleic Acids at Neutral pH," *J.Chinese. Chem. Soc.* **1997**, *44*, 601-07.

[37] (a) Huang, C.Y.; BI, G.X.; Miller, P.S. 'Triplex Formation by Oligonucleotide Containing Novel Deoxycytidine Derivatives," *Nucleic Acids Res.* **1996**, *24*, 2606-13. (b) Hildbrand, S.; Blaser, A.; Parel, S.P.; Leumann, C.J. "5-Substituted 2-Aminopyridine C-Nucleosides as Protonated Cytidine Equivalents: Increasing Efficiency and Selectivity in DNA Triple-Helix Formation," *J. Am. Chem. Soc.* **1997**, *119*, 5499.

[38] Selvasekaran, J.; and Turnbull, K. D. "Chemical Ligation of Oligodeoxynucleotides on Ciruclar DNA Templates," *Nucliec Acids Res.* **1988**, *26*, revision Submitted.

[39] Analysis is conducted with the use of ^{32}P radiolabeled nucleotide components incorporated into the template directed reactions. This allows quantitative analysis of the crude reaction products by densitometry analysis of the autoradiograms from the PAGE runs of reaction aliquots.

[40] Selvasekaran, J.; Cain, III, L.; Turnbull, K.D. "Divalent Metal Effects in Chemical Ligations of Oligodeoxyribonucleotides on Circular DNA Templates," *Manuscript in preparation* for **1998** submission

[41] Selvasekaran, J and Turnbull, K.D. " Analysis of Chemical Reagents For Ligation of Oligodeoxyribonucleotides on Circular DNA Templates," *Nucleic Acids Res.* **1988**, 26, manuscript nearly complete for submission.

[42] Dervan, P.B. "Design of Sequence-Specific DNA-Binding Molecules," *Science*, **1986**, *232*, 464-71.

[43] (a) Wittung, P.; Nielsen, P.; Norden, P.B. "Observation of a PNA-PNA-PNA Triplex," *J. Am. Chem. Soc.* **1997**, *119*, 3189-90. (b) Betts, L.; Josey, J.A. Veal, J.M.; Jordan, S.R. "A Nucleic Acid Triple Helix Formed by a Peptide Nucleic Acid DNA Complex," *Science* **1995**, *270*, 1838-41.(c) Kozlov, I.A.; Nielsen, P.E.; Orgel. L.E. "A method for the P-32 Labeling Of Peptides or Peptide Nucleic Acid Oligomers," *Bioconjugate Chem.* **1998**, *9*, 415-17.

[44] (a) Neilsen, P.E.; Egholm, M.; Buchardt, O. "Sequence-Selective Recognition by DNA by Strand Displacement with a Thymine-Substituted Polyamide," *Science*, **1991**, *254*, 1497-500. (b) Wittung, P.; Nielsen, P.E.; Buchardt, O.; Egholm, M.; Norden, B. "DNA Like Double-Helix Formed by Peptide Nucleic Acid," Nature 1994, *368*, 561-63. (d) Nielsen, P.E.; Haaima, G. "Peptide Nucleic Acid (PNA). A DNA Mimic with a Psuedopeptide Backbone," *Chem. Soc. Rev.* **1997**, 73-78.

[45] Sambrook, T.; Fritsch, E.F.; Maniatis, T. "Molecular Cloning, 2nd Ed. Cold Spring Harbor Laboratory: Cold Spring Harbor, NY," **1989**.

[46] The required matreial will be purchased from Peninsula Laboratories. CA

[47] Saenger, W. "Principles ofd Nucleic Acid Structure," Springer-Verlag: New York, **1984**, 116-158.

[48] The equipment will be purchased from National Labnet Company or through their distributors.

[49] (a) Jaschke, A. " Oligonucleotide Poly(ethylene glycol) Conjugates: Synthesis, Properties and Applications,"*ACS Symposium Series* **1997**, *680*, 265-283. (b) Bonora, G.M.; Ivanova, E.; Zarytova, V.; Burcovich, B.; Veronese, F.M. "Synthesis and Characterization of High-Molecular Mass Polyethylene Gylcol-Conjugated Oligonucleotides," *Bioconjugate Chem.* **1997**, *8*, 793-7.

[50] Herold, D.A.; Keil, K.; Bruns, D.E. "Oxidation of Polyethylene Gylcols By Alcohol Dehydrogenase," *Biochem. Pharmacol.* **1989**, *38*, 73-76.

[51] Shute, R.E.; Rich, D.H. "Synthesis and Evaluation of Novel Activating Mixed Carbonate Reagents for the Introduction of the 2-(TrimethylSilyl) Ethoxy Carbonyl (Treoc) -Protecting Group. Synthesis **1987**, 346-49.

[52] This program was written using Math Lab, 5.0 provided by V MathWorks, Inc.

[53] Stribling, R.; Miller, S.L. "Attempted Nonenzymatic Template-Directed Oligomerizations on a Polyadenylic Acid Template: Implications for the Nature of the First Genetic Material," *J.Mol. Evol.* **1991**, *32*, 282-288.

[54] The required material will be purchased from CruaChem, Inc. VA.

[55] von Kiedrowski, G. "A Self-Replicating Hexadeoxy nucleotide," *Angew. Chem. Int. Ed. Engl.* **1986**, *25*, 932-34.

[56] For modeling the pyr•pur•pyr triplex, an NMR derived solution structure was downloaded from the PDB.[57] The Hoogsteen cytosines were protonated and the resulting triple helical structure was minimized in MacroModel 4.5® using the Polak-Ribier conjugate gradient method with the AMBER force field having water as the solvent. A trimer sequence from the core of this minimized structure which terminated in a 5'-T•A•T triplet and 3'-C•G•C$^+$ triplet (to match template **17**) was excised for modeling the looped linker region connecting the 3'- and 5'-end of the pyrimidine strands of the triplex (Fig. 2). Linker **27** was attached between the 3'- and 5'-end of the pyrimidine strands of the triplex structure. The triplex structure was restricted during this linker minimization step. The linker was minimized with MM2. Time restraints prevented a monte carlo simulation to search for the global minima; however, a molecular dynamics simulation was run after each MM2 to find alternative minima. Further MM2 minimizations (with only the linker unrestricted) were performed to get the best structure in a reasonable timeframe. This entire structure was then restricted while the same linker minimization process was carried out on the other end of the triplex. This process afforded the energy minimized, truncated structure **28**.

[57] (a) Arsensio, J.L.; Brown, T.; Lane, A.N. "Comparison of the Solution Structures of Intramolecular DNA Triple Helices Containing Adjacent and Non-Adjacent GC.C$^+$ Triplets," Private Commun. (b) Abola, E.E.; Sussman, J.L. Prilusky, J.; Manning, N.O. "Protein Data Bank Archives of Three-Dimensional Macromolecular Structures," In: *Methods In Enzymology* (Carter, Jr. C.W.; sweet, R.M, eds.). Academic Press, San Diego. **1997**, *277*, 556-71.

[58] (a) Rumney, IV, S.; Kool, E.T. " Structural Optimization of Non-Nucleotide Loop Replacements for Duplex and Triplex DNAs," *J. Am. Chem. Soc.* **1995**, *117*, 5635-46. (b) Salunkhe, M.; Wu, T.; Letsinger, R.L. "Control of Folding and Binding of Oligonucleotides by Use of a Nonnucleotide Linker," *J. Am. Chem. Soc.* **1992**, *114*, 8768-8722. (c) Benseler, F.; Fu, D.-J.; Ludwig, J.; McLaughlin, L.W. "Hammerhead-Like Molecules Containing Non-Nucleoside Linkers are Active RNA Catalysts,"*J. Am. Chem. Soc.* **1993**, *114*, 8483-84. (d) Thomson, J.B.; Tuschi, T.; Eckstein, F. "Activity of hammerhead Ribozymes Containing Non-Nucleotide Linkers," *Nucleic Acid Res.* **1993**, *21*, 5600-03. (e) Gao, H.; Chidambarm, N.; Chen, B.C.; Pelham, D.E.; Patel, R.; Yang, M.; Zhou, L.; Cook, A.;Cohen, J.S. "Double-Stranded Cyclic Oligonucleotides with Non-Nucleotide Bridges," *Bioconjugate Chem.* **1994**, *5*, 445-543. (f) Ma, M.Y.-X.; Reid, L.S.; Climie, S.C.; Lin, W.C.; Kuperman, R.; Sumner-Smith, M.; Barnett, R.W. "Design and Synthesis of RNA Miniduplexes via a Synthetic Linker Approach," *Biochemistry* **1993**, *32*, 1751-58. (g) Giovannangeli, C.; Momtenay-Garestier,T.; Rougee, M.; Chassignol, M.; Thuong, N.T.; Helen, C,J. "Single Strand DNA as a Target For Triple Helix Formation," *J. Am. Chem. Soc.* **1991**, *113*, 7775-77.

[59] (a) Reynolds, M.A.; Beck, T.A.; Hogrefe, R.I.; Arnold, Jr, L.J.; Vaghefi, M.M. "A Non-Nucleotide-Based Linking Method for the Preparation of Psoralen-Derivatized Methylphosphonate Oligonucleotides," Bioconjugate Chem. 1992, *3*, 366-74. (b) Reynolds, M.A.; Beck, T.A.; Say. P.B.; Schwartz. D.A.; and others."Antisense Oligonucleotides Containing an Internal, Non-Nucleotide-Based Linker Promote Site-Specific Cleavage of DNA," *Nucleic Acids Res.* **1996**, *24*,760-765

[60] Yoo, D.J.; Greenberg, M.M. "Synthesis of Oligonucleotides Containing 3'-Alkyl Carboxylic Acids Using Universal , Photolabile solid Phase Synthesis Supports," *J.Org. Chem.* 1995, *60*, 3358-64.

[61] Asseline, U.; Thoung, N.T. "Synthesis and Properties of Oligonucleotides Covalently Linked to Intercalating Agents," *New. J. Chem.* 1997, *21*, 5-17.

[62] Kayushin, A.L.; Korosteleva, M.D.; Miroshnikov, A.I.; Kosch, W.; Zubov, D.; Piel, N. "A Convenient Approach to the Synthesis of Trinucleotide Phosphoramidities-Synthons for the Generation of oligonucleotide/Peptide Libraries," *Nucleic Acids Res.* **1996**, *24*, 3748-55.

[63] (a) Nielsen, P.E. "Strucutral and Biological Properties of Peptide Nucleic acid (PNA)." *Pure & Appl. Chem.* **1998**, *70*, 105-110. (b) Nielsen, P.E.; Haaima, G. "Peptide Nucleic Acid (PNA). A DNA Mimic with a Psuedopeptide Backbone," *Chem. Soc. Rev.* **1997**, 73-78. (C) Dueholm, K.L.;Nielsen, P.E. "Chemistry, Properties and Applications of PNA (Peptide Nucleic Acid),"*New J. Chem.* **1997**, *21*, 19-31.

[64] (a) Smith, W.T. "Nucleic-Acid Models," *Prog. Polym. Sci.* **1996**, *21*, 209-253. (b) Takemoto, K.; Wada, T.; Chirachanchai, S.; Inaki, Y. "Water Soluble Nucleic Acid Analogs-Preparation and Properties," *Macromolecular Symp.* **1996**, *103*, 119-25. (c) Inaki. Y. "Synthetic Nucleic Acid Analogs," Prog. Polym. Sci. 1992, *17*, 515-566.

[65] (a) Trapane, T.L.; Ts'o. P.O.P. "Triplex Foramtion at Single-stranded Nucleic Acid Target Sites of Unrestricted Sequence by Two Added Strands of Oligonucleotides: A Proposed Model," *J. Am. Chem. Soc.* **1996**, *116*, 10437-49. (b) Rothman, J.H.; Richards, W.G. "Novel Nucleotides Bases for DNA Duplex Recognition by Triple Helix formation," *JCS. Chem. Commun.* **1995**, 1589-90.

The template directed, non-enzymatic polymerization of nucleic acid monomers is not only a model for information transfer systems, but also may potentially be used to direct desired organic reactions in a stereo- and regio-selective manner. The ability of nucleic acid polymers to act as templates can be increased by enhancing base pairing and base stacking of the template-bound complex.

The replication fidelity of the enzymatic polymerization of nucleotide monomers into nucleic acid polymers is an inspiring example in template directed synthesis. Orgel and coworkers have pioneered advances in the template directed nonenzymatic polymerization of chemically activated ribonucleotide monomers. We are investigating methods which allow the chemical ligation of deoxyribonucleotides on DNA templates which are modified to improve the template binding thermodynamics.

CHEMICAL LIGATION/OLIGOMERIZATION OF DNA ON CIRCULAR DNA TEMPLATES

Introduction

Soon after Watson and Crick postulated the model of nucleic acid synthesis directed by a DNA template,[1] Todd aptly expressed the inspiration which this offered to organic synthesis in that it "represents a challenge which must, and surely can, be met by organic chemistry."[2] The preponderance of template-directed reactions reported to date model DNA replication due to its high fidelity and defined molecular contacts. This fidelity is attributed to both intramolecular interactions of nucleic acid bases (stacking) and specific intermolecular interactions (base-paired hydrogen bonding). The combination of these noncovalent forces has directed the design of a variety of template-directed or "artificial replication" systems.[3] The potential for applying the specific interactions of nucleic acid bases to controlled polymerization reactions has been exploited in the non-enzymatic synthesis of RNA oligomers[4] and backbone-modified nucleic acid polymers.[5] Single and double stranded DNA templates have further been utilized for the sequence specific control of ligation, cleavage and crosslinking reactions on the corresponding oligonucleotide recognition sequence.[6]

The potential application of DNA as a defined template for the control of organic reaction asymmetry is limited by the energetics of base pairing/stacking interactions under the desired reaction conditions. These limitations are readily demonstrated by the difficulties encountered in non-enzymatic, DNA template-controlled synthesis of oligonucleotides[4] and backbone-modified nucleic acid polymers.[7]

We have sought to expand the potential usefulness of non-enzymatic DNA template-directed reactions to a variety of organic synthesis problems for a general oligomerization methodology. The goal is to develop a general synthetic methodology with unique chemo-, regio-, and stereoselective potential. The foundational goal has been to initially optimize a template-directed methodology for the ligation and oligomerization of nucleotide derivatives. This required a template-directed process with improved thermodynamic binding of the monomers to the template for better control in reaction selectivity.

Although the Watson-Crick binding and polymerization of nucleotide monomers with template nucleic acid

polymer strands is efficient under enzymatic control, the corresponding non-enzymatic controlled reactions have proved much more challenging.[8] Improved thermodynamic recognition of the nucleobase for the template could be achieved by maximizing the aromatic stacking and number of hydrogen bonding interactions of the template to accessible donor and acceptor positions of a nucleobase. This would increase affinity as well as specificity. As illustrated in Figure 7 , this could most readily be accomplished with a template bound to its nucleobase monomer substrate in both a Watson-Crick as well as a Hoogsteen sense. This would result in a triple helix-like structure. The optimal template for achieving such substrate binding would be circular DNA where the opposite halves cast the antiparallel strands necessary for the Watson-Crick strand and the Hoogsteen strand of a triple helical complex. The substrate nucleobase monomers would then compose the central strand of the triple helix on the circularized DNA template. This should significantly enhance the binding energetics through increased stacking and hydrogen bonding interactions of the monomers with the template relative to a corresponding single-strand template. The improved thermodynamics has been clearly demonstrated by the work of Kool and coworkers using circular DNA to bind single-stranded oligonucleotides.[9]

Figure 7. Ribbon graphic of a circular DNA template with bound monomer nucleobase derivatives and illustration of the template-substrate triplets (**R** represents reacting substrate for oligomerization).

Results and Discussion

Initial studies of template-directed reactions will concentrate on defining the fidelity and regioselectivity of the substrate-template system. Based on the pioneering advancements made by Orgel and coworkers, a ready comparison can be made between template directed reactions on our circularized template, a corresponding single-strand template, and more recent template directed reactions using single-stranded hairpin structures as the template.[10]

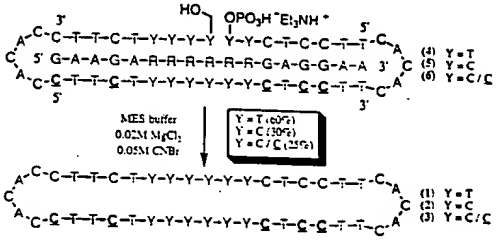
Three different circular DNA templates were designed for our initial studies (**1**, **2** and **3**, Fig. 8). To enforce local structure of the circular DNA template, short primers (**A** and **B**, Fig. 8) with unique sequences were designed as anchoring points for the initiation of triple helix motif interaction of the reacting substrates with the circular template. Propagation through favorable stacking interactions along the template was expected.[11] Each circular template contained the same two primer binding sequences (Fig. 8). Differentiating between the two halves of the template (*i.e.*, the Watson-Crick binding portion and the Hoogsteen binding portion) will control the regioselectivity of the ligations and oligomerizations. For example, forming C^+·GC triplets requires the Hoogsteen C-base to be protonated in order to hydrogen bond to G, while the Watson-Crick C-base is not protonated (*fig.* 7). A 5-methyl-C derivative within a pyrimidine strand is known to increase triple helix formation relative to C due to the lower pK_a.[12] The template was designed with 5-methyl-C's at all Hoogsteen binding portions and C bases at all Watson-Crick binding sites of the template in order to determine if the desired regioselection could be enforced. The ability to propagate the regiospecificity throughout the template-substrate triple helical structure by favorable stacking interactions was to be examined by comparing the ligation and oligomerization results using circular templates **1** and **2** versus **3** (*fig.* **8**). Only **3** would be expected to afford better regioselectivity in the reactions unless the directionality of the oligomer or monomer nucleobase binding to

the template is controlled through propogated stacking from the primers.

Figure 8. Three circular DNA templates (1, 2, and 3) and their respective primers (A and B) for directed ligation and oligomerization experiments. The C designates 5-methyl-cytidine (see text). The highlighted region designates the oligomerization portion of the templates.

The circular DNA templates were synthesized by modifications of existing procedures.[13] After solid-support automated synthesis and purification of oligonucleotides **4**, **5**, and **6** (scheme 15),[14] cyclization was accomplished with cyanogen bromide in the presence of the required purine-rich oligonucleotide for template directed ligation of the two ends. This afforded **1** in 60%, **2** in 30%, and **3** in 25% yield for the cyclization reaction.

Scheme 15



Melting studies were conducted on the circularized templates in order to verify cyclization. An example of the melting analysis is shown in Figure 9. The 11 °C increase in T_m from precircle **4** hybridized to the respective purine-rich oligonucleotide (*fig. 9A*) to template **1** hybridized to the same oligonucleotide (*fig. 9B*), clearly demonstates the successful cyclization to the desired cicular template.

Figure 9. Melting analysis of **4** with the purine-rich oligonucleotide (*9A*) and circularized template **1** with the same oligonucleotide (*9B*).

The hybridization of the primers with the circular templates was also investigated. As shown in Figure 10, single primer association of **B** with template **1** melted at too low a temperature for successful T_m measurement (*fig. 10A*). When both primer **A** and **B** were included with template **1**, a T_m of 12 °C resulted. This suggests a cooperativity in the binding of the two primers to the circular template. With this information, the template directed reactions were generally carried out at ≤ 5 °C.

Figure 10. Melting analysis of circular template **1** with primer **B** (*10A*) and **1** with primers **A** and **B** (*10B*).

A series of ligation reactions were initially to be examined in order to analyze the efficiency of different length oligonucleotides in ligation reactions on the cicularized templates. The 5'-phosphate which would undergo ligation was ^{32}P-labeled in order to allow analysis by PAGE analysis and quantification by autoradiogram

densitometry. As outlined in *figure 5*, the progression from 5' 5-mer oligonucleotide **7** ligating with a 3' 12-mer **8** up to a 5' 12-mer **X** ligating with a 3' 5-mer **X** would be investigated in order to asses the effect of length and directionality of the ligation. The results of this analysis will provide the minimum length needed for the primer in oligomerization studies.

Figure 11. Ligation analysis investigations in order to probe the effect of oligonucleotide length and directionality on the ligation reaction. P* represents the ^{32}P-labeled phosphate which will undergo phosphodiester bond formation.

Initial investigations of 5' 5-mer **7** ligating with 3' 12-mer **8** (5'-^{32}P labeled) were used to develop optimal conditions for the ligation reaction. The results of this initial ligation reaction are shown in Figure 12 . Analysis of the autoradiograms of the PAGE results reveals the ligation reaction was much more efficient on the circular DNA template (lanes 2 (Gel A) and 1 (Gel B)) than on the corresponding single strand template (lanes 1 (Gel A) and 2 (Gel B)). The only ligated products seen in the reactions comigrated with the full length 5'-^{32}P labeled **X** (lane 3 (Gel B)), corresponding to the ligated product of **7** and **8**. Ligation on the single strand template was seen only with $MgCl_2$ in MES buffer. In this case, the ciclular template afforded the ligated product in twice the yield as on the single strand template (62% vs. 32% yield). The ligation reaction was also highly selective with no products arising from the dimerization of the ligating fragments or coupling to the template itself.

Figure 12. Autoradiography of PAGE analysis of the ligation of 5-mer **7** with 5'-^{32}P labeled 12-mer **8**. Gel A shows the results of the reaction run with $NiCl_2$ in imidazole•HCl buffer, while Gel B shows the result of the reaction run with $MgCl_2$ in MES buffer. Lanes 2 (Gel A) and 1 (Gel B) show the migration of the reaction mixture from ligation on circular DNA template **1**. Lanes 1 (Gel A) and 2 (Gel B) compare the results of the ligation reaction on the corresponding single-stamd DNA template **X**. Lane 3 (Gel A) shows the results of the ligation reaction with no template present. Lane 3 (Gel B) shows the migration of the expected full length ligated product which was independently synthesized and 5'-^{32}P labeled.

Conclusion

The sequence-defined, helical structure of DNA makes it an ideal template for the sequence- and stereoselective directing of organic reactions. A circular DNA template has been demonstarted to afford a thermodynamically stable substrate-template complex effective for chemical ligation and oligomerization of deoxy-oligonucleotides and mononucleotides.

We realized the current limitation of this template directed methodology to the two purine nucleobase substrates, A and G, due to the present inability to efficiently recognize pyrimidines *via* triple helix formation.

Future investigations will attempt to overcome this limitation by taking advantage of known *novel nucleobases* for recognition and incorporation into the circular template.[15]

Materials and methods

Phosphoramidites, solid supports and chemicals for DNA synthesis were obtained from either Cruachem Inc., or Penninsula Laboratories. All enzymes were purchased from Boehringer-Mannheim (or) New England Biolabs. The radiolabelled 5'-(α-^{32}P) ATP (>6000Ci/mmol) was obtained from Amersham. . Analytical and preparative HPLC was performed with a Schimadzu LC-600 Liquid Chromatograph with SPD-6A UV spectophotometric detector using Varian 150 x 4.6mm, 5m, C18, 90A column . Oligomers were also purified by preparative 20% denaturing polyacrylamide gel electrophoresis, desalted (using Sep-Pak^R from Waters) and quantitated using Hitachi U-2000 spectrophotometer by absorbance at 260 nm. Molar extinction coefficients for the oligomers were calculated by the nearest neighbor method.[16] The melting studies were carried out in teflon-stoppered 1cm path length Quartz cells under nitrogen atomsphere on a Jasco-710 spectropolarimeter equipped with Jasco programmable temperature control. Absorbance(260nm) was monitored while temperature was raised at a rate of 0.5 ^{0}C/min. The scintillation counting was done on a Wallac 1410 Liquid Scintillation Counter. All other Chemicals were of analytical grade (or) HPLC grade. Standard molecular biology techniques were used, if not mentioned otherwise.[15] DNA oligonucleotides were synthesized on an Applied Biosystems 392 synthesizer using β- cyanoethyl phosphoramidite chemistry.[15] 3'-phosphate ends were synthesized using the modified solid supports purchased from Penninsula Laboratories. Circularization of linear 3'-phosphorylated pre circle oligomer was achieved using short oligonucleotide templates to align the reactive ends and BrCN/MES(Et_3N) buffer pH=7.5/Mg^{2+} chemistry to achieve the ligation.[14]

Thermal denaturation studies

Solutions for the thermal denaturation studies contained a one to one ratio of the circular oligomer and the complementary oligomer(3mM each). Also present were 100mM NaCl and 10mM $MgCl_2$. Solutions were buffered with 10mM MES(Et_3N) at pH 7.5. After the solutions were prepared they were heated to 90 ^{0}C and allowed to cool slowly to room temperature prior to the melting experiments.

Preparation of ^{32}P-labelled oligomers

10pmol of the gel purified oligomer was dissolved in 10.4ml of sterilized water, 2ml of 10x kinase buffer,6ml of (α -^{32}P) ATP (60mCi) and 2ml of T_4-polynucleotide kinase were then added for a total volume of 21.4ml. Following incubation at 37 ^{0}C for 1.5-2h, the reaction mixture was heated at 70 ^{0}C for 5 min and slowly cooled to room temperature, purified by Sep-Pak chromatography.

Polymerization reaction conditons

Reactions were carried out according to the several published literature procedures[11,14]. Autoradiographic detection were used to analyze the product formation. Product yields were estimated from densitometry analysis of scanned images of autoradiograms.

References

(1) (a) Watson, J. D.; Crick, F. H. C. *Nature* **1953**, *171*, 737. (b) Crick, F. H. C.; Watson, J. D. *Proc. R. Soc. London (Ser. A)* **1954**, *223*, 80

(2) Todd, A. in *Perspectives in Organic Chemistry*, Todd, A., Ed.; Interscience, New York, **1956**, p. 245.

(3) For relevant reviews see: (a) Hoffmann, S. *Angew. Chem. Int. Ed. Engl.* **1992**, *31*, 1013. (b) Lindsey, J. S. *New J. Chem.* **1991**, *15*, 153. (c) Philp, D.; Stoddart, J. F. *Synlett* **1991**, 445. (d) Anderson, S.; Anderson, H. L.; Sanders, K. M. *Acc. Chem. Res.* **1993**, *26*, 469-475.

(4) Orgel, L. E. *Nature* **1992**, *358*, 203.

(5) Inaki, Y. *Prog. Polym. Sci.* **1992**, *17*, 515.

(6) Leading references: (a) Ligation: (*i*) Goodwin, J. T.; Lynn, D. G. *J. Am. Chem. Soc.* **1992**, *114*, 9197. (*ii*) Luebke, K. J.; Dervan, P. B. *J. Am. Chem. Soc.* **1989**, *111*, 8733. (b) Cleavage and crosslinking: (*i*) Kido, K.; Inoue, H.; Ohtsuka, E. *Nucleic Acids Res.* **1992**, *20*, 1339. (*ii*) Nielsen, P. E. *Bioconjugate Chem.* **1991**, *2*, 1. (iii) Coleman, R. S.; Kesicki, E. A. *J. Org. Chem.* **1995**, *60*, 6252-6253. (iv) Woo, J.; Hopkins, P. B. *J. Am. Chem. Soc.* **1991**, *113*, 5457- 5459.

(7) For a brief overview, see: ref. 3(b), p. 166. For more specific discussion of each area see ref. 4 and ref. 5 and references cited therein.

(8) Joyce, G. F. *Cold Spring Harbor Symposium on Quantitative Biology*, Cold Spring Harbor, NY, 1987; Vol. LII, pp 41-51.

(9) Kool, E. T. *Chem. Rev.* **1997**, *97*, 1473-1487.

(10) (a) Wu, T.; Orgel, L. E. *J. Am. Chem. Soc.* **1992**, *114*, 7963. (b) James, K. D.; Ellington, A. D. *Chemistry and Biology*. **1997**, *4*, 595-605.

(11) See: Colocci, N.; Distefano, M. D.; Dervan, P. B. *J. Am. Chem. Soc.* **1993**, *115*, 4468, and references cited.

(12) See: Plum, G. E.; Park, Y.-W.; Singleton, S. F.; Dervan, P. B.; Breslauer, K. J. *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 9436, and cited references. Alternatively, the use of "permanently protonated"-C mimics such as pseudoisocytidine (Ono, A.; Ts'o, P. O. P.; Kan, L.-s. *J. Org. Chem.* **1992**, *57*, 3225.) or other non-natural deoxyribonucleosides (Koh, J. S.; Dervan, P. B. *J. Am. Chem. Soc.* **1992**, *114*, 1470.) may be employed for directing Hoogsteen hydrogen bonding in future studies.

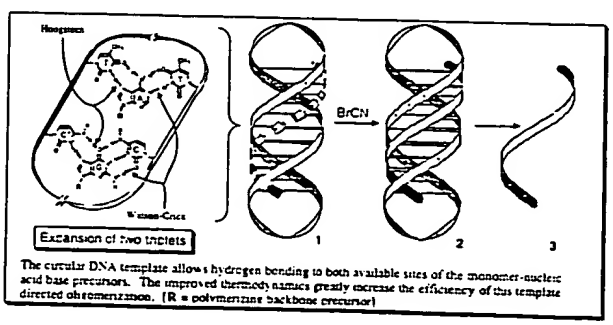
(13) (a) Kanaya, E.; Yangawa, H. *Biochemistry*. **1986**, *25*, 7423-7430. (b) Dolinnaya, N. G.; Blumenfeld, M.; Merenkova, I. N.; Oretskaya, T. S.; Krynetskaya, N. F.; Ivanovskaya, M. G.; Vassuer, M.; Shabarova, Z. A. *Nucliec Acids Research*. **1993**, *21*, 5403-5407.

(14) (a) Sambrook, T.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning* 2nd Ed. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989. (b) Beaucage, S. L.; Caruthers, M. H. *Tetrahedron Letters* **1981**, *22*, 1859.

(15) For a general reference to several possible combinations of novel nucleobases in triple helix formation, see: Trapane, T.L.; Ts'O, P.O.P. *J. Am. Chem. Soc.* **1994**, *116*, 10437-49.

(16) Boren, P. N. (1985) in Fasman, G. D, (ed.), Handbook of Biochemsitry and Molecular Biology, 3rd Ed, CRC press, Cleveland, Vol *1* 585.

Scheme 16



The circular DNA template allows hydrogen bonding to both available sites of the monomer-nucleic acid base precursors. The improved thermodynamics greatly increase the efficiency of this template directed chemistry. (R = polymerizing backbone precursor)

An Economical and Environmentally Friendly Approach to the Large-Scale Synthesis of Therapeutically Active Oligonucleotides

Therapeutic treatment of human disease is poised to undergo significant advancement with ever increasing knowledge of the human genome. The emphasis of genome research is shifting from mapping to sequencing and functional analysis.[1] Converting the knowledge of genomic function into disease treatment requires the chemical means to recognize and regulate genetic transformations. One of the most direct means to mediate genetic transformation is through antigene approaches.[2] Antigene methods involve the use of natural or non-natural oligonucleotides for sequence-specific binding to double helical DNA.[3] This can be used to block transcription or as a means to modify the target genetic sequence.[2]

Although antigene therapeutics holds a great deal of promise for a general approach in treating diseases at the genetic level, research remains to be accomplished in order to bring the proven technology to a commercially viable therapeutic method. One of the key limitations is the ability to produce large scale (multi-gram) quantities of desired natural or non-natural oligonucleotides. The state-of-the-art techniques for oligonucleotide synthesis through well established automated, solid-phase chemistry is still limited to the low millimole scale on a routine basis.[4] The impracticalities of solid-support chemistry and the environmental impact of high volume waste generated by multistep synthesis of oligomers on larger scales is quickly realized.

We are rapidly progressing in the development of a methodology for natural and non-natural oligonucleotide synthesis in a single chemical step which produces little waste and environmentally inert by-products. The chemistry can be accomplished under aqueous conditions from cheap, commercially available starting monomers which are stable and require no protecting groups for the oligomerization process. This is in contrast to the present solid-phase methodology requiring a minimum of eight chemical steps per monomer addition, expensive starting materials which are hydrolytically unstable, uneconomical use of multiple protecting groups, and utilizes environmentally harmful solvents and reagents producing significant waste.[5]

We have found the use of a small circularized DNA as a template in oligomerization of commercially available 5'-monophosphate nucleotide salts offers greatly enhanced yields over reactions on single-strand DNA templates (see scheme 2). The mononucleotides bind to the circularized template to form a triple helix-like structure (**1**). The improved thermodynamics of this monomer-template complex allow for efficient oligomerization in buffered water with cyanogen bromide to produce the sequence-defined oligonucleotide (**2**). The product (**3**) is readily washed from the template. The inert by-products are ammonia, carbon dioxide and sodium bromide. The synthesis of a standard 15-base, purine-rich oligonucleotide (0.2 micromole scale) would be approximately 1,000-fold cheaper than using the conventional phosphoramidite, solid-phase synthetic method. Expansion of this methodology to multigram scales will be accomplished by attachment of the circular DNA template to a solid support for repeated cycles of aqueous based oligomerization reactions. Modified backbone oligonucleotide derivatives will be prepared from the appropriate monomer precursors.

The 1,000-fold savings in material costs and alleviation of waste concerns will assure certain commercial success for the large-scale synthesis of therapeutically active oligonucleotides.

References

(1) For a complete resource covering many aspects of the human genome see the Human Genome Database (GDB) hosted at Johns Hopkins University (http://www.gdb.org/): Letovsky, S.I.; Cottingham, R.W.; Porter, C.J.; Li, P.W.D. "GDB: The Human Genome Database," *Nucleic Acids Res.* **1998**, *26*, 94-99.
(2) For a selection of recent examples, see: (a) Matteucci, M.; Lin, K.-Y.; Huang, T.; Wagner, R.; Sternbach, D.D.; Mehrotra, M.; Besterman, J.M. "Sequence-Specific Targeting of Duplex DNA Using a Camptothecin-Triple Helix Forming Oligonucleotide Conjugate and Topoisomerase I," *J. Am. Chem. Soc.* **1997**, *119*, 6939-40. (b) Faruqi, A.F.; Seidman, M.M.; Segal, D.J.; Carroll, D.; Glazer, P.M. "Recombination Induced by Triple-Helix-Targeted DNA Damage in Mammalian Cells," *Mol. and Cell. Biol.* **1996**, *16*, 6820-28. (c) Bouziane, M.; Cherny, D.I.; Mouscadet, J.-F.; Auclair, C. "Alternate Strand DNA Triple Helix-mediated Inhibition of HIV-1 U5 Long Terminal Repeat Integration *in Vitro*," **1996**, *271*, 10359-364.
(3) For a recent review on triple helix mediated DNA recognition, see: Doronina, S.O.; Behr, J.-P. "Toward a General Triple Helix Mediated DNA Recognition Scheme," *Chem. Soc. Rev.* **1997**, 63-71.
(4) For select reviews see: (a) Beaucage, S.L.; Iyer, R.P. "Advances in the Synthesis of Oligonucleotides by the Phosphoramidite Approach," *Tetrahedron* **1992**, *48*, 2223-2311. (b) Hobbs, J.B. "Nucleotides and Nucleic Acids," *Organophosphorus Chemistry* **1990**, *21*, 201-321. (c) Sonveaux, E. "The Organic Chemistry Underlying DNA Synthesis," *Bioorg. Chem.* **1986**, *14*, 274-325.
(5) For standard protocol in the conventional phosphoramidite synthesis, see: (a) Beaucage, S.L.; Caruthers, M.H. *Tetrahedron Lett.* **1981**, *22*, 1859. (b) Sinha, N.D.; Biernat, J.; McManus, J.; Koster, H. *Nucleic Acids Res.* **1984**, *12*, 4539-57.

The enzyme controlled synthesis of nucleic acids directed by a DNA template is the model of fidelity and regiospecificity for designing template-directed reactions in organic synthesis. This research has focused on the design, synthesis and evaluation of methodology for template-directed chemical ligations and oligomerizations of DNA oligonucleotides and mononucleotides. The use of circularized DNA as the template for directing the assembly of desired reaction substrates has been investigated. The effect of primers, primer lenngth, concentrations, buffer, salts, pH, temperature, coupling reagent, and coreagents has been investigated. The difference between ligation of 3'-phosphates and 5'-phosphates has also been investigated. The present optimal conditions for the ligation and oligomerization reactions has resulted. In all cases investigated to date, the ligation of short DNA oligonucleotides on circularized DNA templates is always more efficient than the corresponding ligations on single-strand Watson-Crick DNA templates.

Use of Circular Oligomer Templates for Directing Chemical Reactions

The use of DNA or RNA as templates upon which substrates bind for chemically induced covalent bond formation has been known since 1966.[1] Since that time numerous oligonucleotide ligation reactions have been reported in duplex directed systems with single strand DNA templates, where Watson-Crick hydrogen bonding affords the substrate-template association.[2] ODN ligations have also been reported in triplex directed systems with double strand templates, where Hoogsteen hydrogen bonding of homopyrimidine ligation substrates to the homopurine strand of a homopryrimidine-homopurine Watson-Crick duplex affords the substrate-template complex.[3] Non-enzymatic, template directed ligation strategies are particularly advantageous for constructing non-natural, modified oligonucleotides.[4] This includes

---

[1] Naylor, R.; Gilham, P.T. ÒStudies on Some Interactions and reactions of Oligonucleotides in Aqueous Solution,Ó *Biochemistry* **1966**, *5*, 2722-28.

[2] (a) Kanaya, E.; Yanagawa, H. ÒTemplate-Directed Polymerization of Oligoadenylates Using Cyanogen Bromide,Ó *Biochemistry* **1986**, *25*, 7423-30. (b) Zuber, G.; Sirlin. C.; Behr, J.P. ÒEnhanced Ligation of DNA with a Effector Molecule,Ó *J. Am. Chem. Soc.* **1993**, *115*, 4939-40. (c) Dolinnaya, N.G.; Sharbova, Z.A. ÒChemical Ligation as a Method for the Assembly of Double-Stranded Nucleic Acids: Modifications and Local Structure Studies,Ó *Russ. Chem. Bull.* **1996**, *45*, 1787-1809.

[3] (a) Luebke, K.J.; Dervan, P.B. ÒNonenzymatic Ligation of Oligodeoxyribonucleotides on a Duplex DNA Template by Triple-Helix Formation,Ó *J. Am. Chem. Soc.* **1989**, *111*, 8733-35. (b) Luebke, K.J.; Dervan, P.B. ÒNonenzymatic Ligation of Double-Helical DNA by Alternate-Strand Triple Helix Formation,Ó *Nucleic Acids Res.* **1992**, *20*, 3005-09. (c) Dolinnanya, N.G.; Pyatarauskene, O.V.; Shabarova, Z.A. ÒProbing DNA Triple Helix Structure by Chemical Ligation,Ó *FEBS Lett.* **1991**, *284*, 232-34.

[4] (a) Zeilinski, W.S.; Orgel, L.E. ÒAuto Catalytic Synthesis of a Tetra Oligonucleotide Analogue,Ó*Nature* **1987**, *327*, 346-47. (b) von Kiedrowski, G.; Wlotzka, B.; Helbing, J.; Matzen, M.; Jordan, S. ÒParabolic Growth of a Self-Replicating Hexadeoxynucleotide Bearing a 3Õ-5Õ-Phosphoamidate Linkage,Ó *Angew. Chem. Int. Ed. Engl.* **1991**, *30*, 423-26. (c) Dolinnaya, N.G.; Sokolova, N.I.; Ashirbekova, D.T.; Shabarova, Z.A. Ò*Nucleic Acids Res.* **1991**, *19*, 3067-72. (d) Rumney IV, S.; Kool, E.T. ÒDNA Recognition by Hybrid Oligoether-Oligodeoxynucleotide Macrocycles,Ó *Angew. Chem. Int. Ed. Engl.* **1992**, *31*, 1617-19. (e) Luo, P.; Leitzel. J.C.; Zhan, Z.-Y.J.; Lynn, D.G. ÒAnalysis of the Structure and Stability of a Backbone-Modified Oligonucleotide: Implications for Avoiding Product Inhibition in Catalytic Template-Directed Synthesis,Ó *J. Am. Chem. Soc.* **1998**, *120*, 3019-31. (f) Sawai, H.; Totsuka, S.; Yamamoto, K.; Ozaki, H. ÒNon-Enzymatic, Template-Directed Ligation of 2Õ-5Õ Oligoribonucleotides. Joining of a Template and a Ligator Strand,Ó *Nucleic Acids Res.* **1998**, *26*, 2995-3000. (g) Herrlein, M.K.; Letsinger, R.L. ÒSelective Chemical Autoligation on a Double-Stranded DNA Template,Ó *Nucleic Acids Res.* **1994**, *22*, 5076-78. (h) Bohler, C.; Nielsen, P.E.; Orgel, L.E. ÒTemplate Switching Between PNA and RNA Oligonucleotides.Ó *Nature* **1995**, *376*, 578-81. (i) Pitsch, S.; Krishnamurthy, R.; Bolli, M.; Wenderborn.S.; Holzner, A.; Minton, M.; Lesueur, C.; Schlšnvogt, I.; Jaun, B.; Eschenmoser, A. ÒPyranosyl-RNA (Òp-RNAÕ): Base-Pairing Selectivity and Potential to Replicate,Ó *Helv. Chim. Acta.* **1995**, *78*, 1621-34.(j) Schmidt, J.G.: Christensen, L.; Nielsen, P.E.; Orgel, L.E. ÒInformation Transfer from DNA to Peptide Nucleic Acids by Template-Directed Syntheses,Ó *Nucleic Acids Res.* **1997**, *25*, 4792-96. (k) Bruick, R.K.; Dawson, P.E.; Kent, S.B.H.; Usman, N.; Joyce, G.F. ÒTemplate-Directed Ligation of Peptides to Oligonucleotides,Ó *Chem. & Biol.* **1996**, *3*, 49-56. (l) Gryanzov, S.M.; Letsinger, R.L. ÒChemical Ligation of Oligonucleotides in the Presence and Absence of a Template,Ó *J. Am. Chem. Soc.* 1993, *115*, 3808-09.

the synthesis of small, circular DNA through the template directed circularization of linear ODNs.[5]

Chemically activated, template directed ligation and oligomerization reactions have gained interest for their potential role in prebiotic DNA and RNA synthesis.[6,7,8] The higher association of short ODNs with DNA templates has resulted in numerous

---

[5] (a) Prakash, G.; Kool, E.T. ÒStructural Effects in the Recognition of DNA by Circular Oligonucleotides,Ó *J. Am. Chem. Soc.* **1992**, *114*, 3523-27. (b) Rubin, E.; Rumney IV. S.; Wang, S.; Kool, E.T. ÒConvergent DNA Synthesis: A Non-Enzymatic Dimerization Approach to Circular Oligodeoxynucleotides,Ó *Nucleic Acids Res.* **1995**, *23*, 3547-53. (c) Li, T.; Weinstein, D.S.; Nicolaou, K.C. ÒThe Chemical End-Ligation of Homopyrimidine Oligodeoxyribo-nucleotides within a DNA Triple Helix,Ó *Chem. & Biol.* **1997**, *4*, 209-14. (d) Dolinnaya, N.G.; Blumenfeld, M.; Merenkova, I.N.; Oretskaya, T.S.; Krynetskaya, N.F.; Ivanovskaya, M.G.; Vasseur, M.; Shabarova, Z.A. ÒOligonucletoide Circularization by Template-Directed Chemical Ligation,Ó *Nucleic Acids Res.* **1993**, *21*, 5403-07. (e) Herrlein, M.K.; Nelson, J.S.; Letsinger, R.L. ÒA Covalent Lock for Self-Assembled Oligonucleotide Conjugates,Ó *J. Am. Chem. Soc.* **1995**, *117*, 10151-52. (f) Xu, Y.; Kool, E.T. ÒChemical and Enzymatic Properties of Bridging 5Õ-S-Phosphorothioester Linkages in DNA,Ó *Nucleic Acids Res.* **1998**, *26*, 3159-64.

[6] (a) Li, T.; Nicolaou, K.C. ÒChemical Self-Replication of Palindromic Duplex DNA,Ó *Nature* **1994**, *369*, 218-21. (b) Bag, B.G.; von Kiedrowski, G. ÒTemplates. Autocatalysis and Molecular Replication,Ó *Pure & Appl. Chem.* **1996**, *68*, 2145-52. (c) Rohatgi, R.; Bartel, D.P.; Szostak, J.W. ÒKinetic and Mechanistic Analysis of Nonenzymatic Template-Directed Oligoribonucleotide Ligation,Ó *J. Am. Chem. Soc.* **1996**, *118*, 3332-39. (d) Bolli, M.; Micura, R.; Eschenmoser, A. Ò Pyranosyl RNA: Further Observations On Replication,Ó *Chem. & Biol.* **1997**, *4*, 309-20.

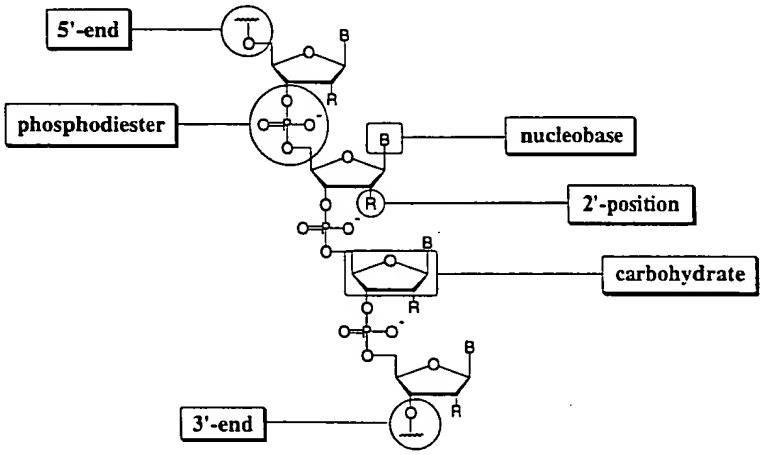
[7] (a) Wu, T.; Orgel, L.E. ÒNonenzymatic Template-Directed Synthesis on Oligodeoxycytidylate Sequences in Hairpin Oligonucleotides,Ó *J. Am .Chem. Soc.* **1992**, *114*, 317-22. (b) Wu, T.; Orgel, L.E. ÒNonenzymatic Template-Directed Synthesis on Hairpin Oligonucleotides. 2. Templates Containing Cytidine and Guanosine Residues,Ó *J. Am .Chem. Soc.* **1992**, *114*, 5496-501. (c) Wu, T.; Orgel, L.E. ÒNonenzymatic Template-Directed Synthesis on Hairpin Oligonucleotides. 3. Incorporation of Adenosine and Uridine Residues,Ó *J. Am .Chem. Soc.* **1992**, *114*, 7964-69.(d) Schmidt, J.G.; Nielsen, P.E.; Orgel, L.E. ÒInformation Transfer from Peptide Nucleic Acids to RNA by Template-Directed Syntheses,Ó *Nucleic Acids Res.* **1997**, *23*, 4797-802. (e)Rodriguez, L.; Orgel. L.E. ÒTemplate-Directed Extension of a Guanosine 5Õ-Phosphate Covalently Attached to an OligoDeoxycytidylate Template,Ó *J.Mol. Evol.* **1991**, *33*, 477-82. (f) Rembold, H.; Orgel, L.E. ÒSingle-Strand Regions of Poly (G) Act as Templates for Oligo (C) Synthesis, *J.Mol. Evol.* **1994**, *33*, 205-10. (g) Ertem, G.; Ferris, J.P. ÒSynthesis of RNA Oligomers on Heterogeneous Templates,Ó *Nature* **1996**, *379*, 238-40. (g) Schwartz, A.W.; Orgel, L.E. ÒTemplate-Directed Synthesis of Novel, Nucleic Acid-Like Strucures,Ó *Science* **1985**, *228*, 585-587. (h) Stribling, R.; Miller, S.L. ÒTemplate-Directed Synthesis of Oligonucleotides Under Eutectic Conditions, Ò *J.Mol. Evol.* **1991**, *32*, 289-95. (h) Kurz, M.; Gšbel, K.; Hartel, C.; Gšbel, M.W. ÒNonenzymatic Oligomerization of Ribonucleotides on Guanosine-Rich Templates: Suppression of the Self-Pairing of Guanosine,Ó *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 842-45. (I) Prakash, T.P.; Roberts, C.; Switzer, C. ÒActivity of 2Õ5Õ-Linked RNA in the Template-Directed Oligomerization of Mononucleotides,Ó *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 1522-23.

[8] (a) Kanavarioti, A. ÒSelf-Replication of Chemical Systems Based on Recognition Within a Double or a triplex- A Realistic Hypothesis,Ó *J. Theor. Biol.* **1992**, *158*, 207-19. (b) Kanavarioti, A. ÒTemplate-Directed Chemistry and the Origins of the RNA World,Ó *Orig. Life Evol. Biosphere* **1994**, *24*, 479-94. (c) Inoue, T.; Orgel, L.E. Ò A Nonenzymatic RNA Polymerase Model,Ó *Science* **1983**, *219*, 859-62. (d) Orgel, L.E.; Lohrmann, R. ÒPrebiotic Chemistry and Nucleic Acid Replication,Ó *Acc. Chem. Res.* **1974**, *7*, 368-377.

reports of template directed ligation reactions of short ODNs as a less challenging alternative to template directed mononucleotide oligomerizations.[9]

The present circular templates and methods inlude any of the approaches used for linear template directed reactions noted, but modified by circularizing the template such that the template will display recognition elements on opposing sides of the circular template for complexation with the substrates undergoing reaction. The present method includes modification of the oligonucleotide circular template for broader applications of template control in directing a variety of chemical reactions under a broad range of conditions. These extensions include the use of any circular oligomer which will display the binding elements for template-substrate association for the purpose of directing or controlling covalent bond formation of the substrates. This includes any standard modifications of nucleic acid components (Figure 13) such as described in various scientific literature reviews.[10],[11],[12]

Standard Modifications of Nucleic Acid Oligomers



(10) Beaucage, S.L.; Iyer, R.P. *Tetrahedron* **1993**, *49*, 1925-63.
(11) Uhlmann, E.; Peyman, A. *Chem Rev.* **1990**, *90*, 543-84.

Figure 13 . Components of nucleic acid polymers that are commonly modified to induce selective properties or functionality to an oligomer.

[9] (a) Harada, K.; Orgel, L.E. ÒIn Vitro Selection of Optimal DNA Substrates for Ligation by a Water-Soluble Carbodiimide,Ó *J. Mol. Evol.* **1994**, *38*, 558-60. (b) James, K. D.; Ellington, A.D. ÒSurprising Fidelity of Template-Directed Chemical Ligation of Oligonucleotides,Ó *Chem. & Biol.* **1997**, *4*, 595-605.

[10] Beaucage, S. L. and Iyer, R. P. ÒThe Functionalization of Oligonucleotides Via Phosphoramidite Derivatives,Ó *Tetrahedron* **1993**, *49*, 1925-63.

[11] Uhlmann, E. and Peyman, A. ÒAntisense Oligonucleotides: A New Therapeutic Principle,Ó *Chem. Rev.* **1990**, *90*, 543-84

[12] Varma, R. S. ÒSynhtesis of Oligonucleotide Analogues with Modified Backbones,Ó *Synlett* **1993**, 621-37.

The present invention also includes, but is not limited to, modifying the deoxyribonucleotide template at selective backbone positions (such as the 2Õ-positions of the ribose) with ionized subtituents which alter the overall charge of the circular template and with sterically modifying substituents or binding moieties to alter the asymmetry and binding recognition capabilities of the circular template. Modification of the backbone is made to alter stability, solubility and substrate binding capabilities of the circular template. These include a range of alterations from simple changes in the phosphodiester moiety by replacing oxygen phosphate linkages to other heteroatom phosphate linkages (*i.e.*, nitrogen and sulfur) to complete replacement of the phosphodiester with other suitable linkages. These modifications also include changes in the ribose/deoxyribose component of the template backbone to other carbohydrates or any moiety which will allow the display of a nucleobase heterocycle or any such hydrogen bonding or template-substrate binding component. Modifications to the backbone further include complete replacement of the ribo-/deoxyribophosphodeister backbone with alternative oligomers such as peptide and peptide mimics,[13] or other oligomers such as polyolefins (Figure 14) (including polyacrylates, polyacrylamides, polystyrenes), polyethers, polyamides or any such polymers which will allow the display of substrate binding moieties in a circular template for directing chemical reactions.[14] These types of modified oligomers include all those described in several scientific literature reviews (hereby incorporated by reference).[15],[16],[17],[18] Modification of the nucleic acid heterocycle components is also included in order to expand the number of substrates which can be complexed with the circular template for directing covalent bond formation between bound substrates.[10,11,19] These will include the incorporation of known modified bases which have been designed specifically for applications in triplex DNA formation (hereby incorporated by reference).[20],[21]

---

[13] (a) Nielsen, P.E. ÒStrucutral and Biological Properties of Peptide Nucleic acid (PNA).Ó *Pure & Appl. Chem.* **1998**, *70*, 105-110. (b) Nielsen, P.E.; Haaima, G. ÒPeptide Nucleic Acid (PNA). A DNA Mimic with a Psuedopeptide Backbone,Ó *Chem. Soc. Rev.* **1997**, 73-78. (c) Dueholm, K.L.;Nielsen, P.E. - ÒChemistry, Properties and Applications of PNA (Peptide Nucleic Acid),Ó*New J. Chem.* **1997**, *21*, 19-31.

[14] (a) Smith, W.T. ÒNucleic-Acid Models,Ó *Prog. Polym. Sci.* **1996**, *21*, 209-253. (b) Takemoto, K.; Wada, T.; Chirachanchai, S.; Inaki, Y. ÒWater Soluble Nucleic Acid Analogs-Preparation and Properties,Ó *Macromolecular Symp.* **1996**, *103*, 119-25. (c) Inaki. Y. ÒSynthetic Nucleic Acid Analogs,Ó Prog. Polym. Sci. 1992, *17*, 515-566.

[15] Inaki, Y. ÒSynthetic Nucleic Acid Analogs,Ó *Prog. Polym. Sci.* **1992**, *17*, 515-70.

[16] Akashi, M. and Takemoto, K. ÒNew Aspects of Polymer Drugs,Ó In *Adv. Polym. Sci.* **1990**, *97*, 107-46.

[17] Inaki, Y. and Takemoto, K. ÒFunctionality and Applicability of Synthetic Nucleic Acid Analogs,Ó In *Curr. Top. Polym. Sci.* **1988**, *1*, 79-100.

[18] Pitha, J. ÒPhysiological Activities of Synthetic Analogs of Polynucleotides.Ó In *Adv. Polym. Sci.* **1983**, *50*, 1-16.

[19] (a) Trapane, T.L.; TsÕo. P.O.P. ÒTriplex Foramtion at Single-stranded Nucleic Acid Target Sites of Unrestricted Sequence by Two Added Strands of Oligonucleotides: A Proposed Model.Ó *J. Am. Chem. Soc.* **1996**, *116*, 10437-49. (b) Rothman, J.H.; Richards, W.G. ÒNovel Nucleotides Bases for DNA Duplex Recognition by Triple Helix formation.Ó *JCS. Chem. Commun.* **1995**, 1589-90.

[20] (a) Ono, A.; TsÕO, P.O.P.; Kan, L.S. ÒTriplex Formation of an Oligomer Containing 2Õ-O-Methyl Pseudo IsoCytidine With a DNA Duplex at Neutral pH,Ó *J.Org.Chem.* **1992**, *57*, 3225-30. (b) Ono,

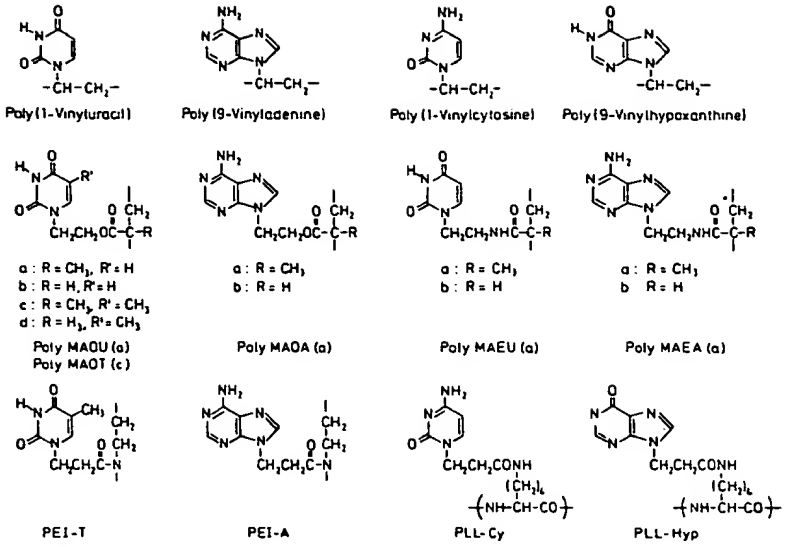


Figure 14. Synthetic Nucleic Acid Analogs.

The present templates and methods include the use of a variety of different monomer (Figure 14) and higher multimer components as substrates for ligation and multi-ligation up to polymerization and copolymerization for covalent bond forming reactions conducted in the presence of the circular templates for controlling the reactions.

The present invention encompasses modifications to make the template more stable including any modification that will allow the molecule, compound, or the like to bind to two sides of a circular template to increase, improve, facilitate, or cause substrate binding.

A.; TsÕO, P.O.P.; Kan, L.S. ÒTriplex Formation of an Oligomer Containing 2Õ-O-Methyl Pseudo IsoCytidine With Single and Double Stranded Nucleic Acids at Neutral pH.Ó *J.Chinese. Chem. Soc.* 1997, *44*, 601-07.

[21] (a) Huang, C.Y.; BI, G.X.; Miller, P.S. ÔTriplex Formation by Oligonucleotide Containing Novel Deoxycytidine Derivatives.Ó *Nucleic Acids Res.* 1996. *24*, 2606-13. (b) Hildbrand, S.; Blaser, A.; Parel, S.P.; Leumann, C.J. Ò5-Substituted 2-Aminopyridine C-Nucleosides as Protonated Cytidine Equivalents: Increasing Efficiency and Selectivity in DNA Triple-Helix Formation,Ó *J. Am. Chem. Soc.* 1997, *119*, 5499.